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THE INTERPRETATION OF ESTROGENIC ASSAYS

JACK M. CURTIS, ERNEST J. UMBERGER, AND LILA F. KNUDSEN

From the Division of Pharmacology and Food Division, Food and Drug Administration, Federal Security Agency

WASHINGTON, D. C.

ALTHOUGH the assay procedures for the determination of estrogenic potency by the vaginal smear technique are well established, and vary from laboratory to laboratory only in minor details, the interpretation of the results of these assays is often open to question. The matching of the responses to the standard and unknown as contemplated by the earlier writers (reviewed by Gustavson, 1939) is a matter of chance and when these responses are not exactly equivalent the estimation of potency becomes dependent on the judgment of the assayist. Further, the only measure of the accuracy of these determinations is their reproducibility, and the evaluation of this characteristic is frequently more subjective than objective.

Objective methods of analysis have been supplied by the statistical procedures developed by Bliss (1935) and by Irwin (1937). However, objections to their general use have been raised by assayists on the grounds that the calculations required are complicated, tedious, and difficultly understood by the statistically-untrained assayist. Also, the availability of a calculating machine is almost a necessity. These objections are directed against the use of a cumbersome tool and not against the end attained by its use. The advantages of having an objective means of interpretation and estimation of the accuracy of an assay are obvious.

Our efforts have been directed toward the development of a rapid method of statistical analysis applicable to estrogenic assay results that will require only very few and simple calculations and will give an interpretation in terms of potency as percent of the reference standard and also standard error of the assay in the same terms. This

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has been accomplished and the mathematical derivations and implications have been published elsewhere (Knudsen and Curtis, 1947). This report gives the details of its application to estrogenic assays.

METHOD OF ASSAY

The preparation of the rats for assay, the care of the assay colony and the method of making and evaluating the vaginal smears have been described previously by Curtis, Witt, and Knudsen (1944). All solutions were in corn

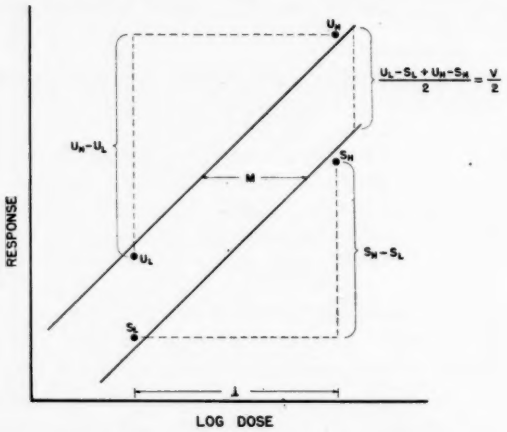

FIG. 1. Graph of typical two dose assay.

oil and injections of a constant volume (0.1 cc.) were subcutaneous. The total dose of 0.2 cc. was divided into 2 equal parts injected at a 7-hour interval rather than 3 parts administered at a 4½-hour interval as previously used in this laboratory. The adoption of this labor-saving device was supported by the results of Pugsley and Morrell (1943) who showed that the slope of the dosage-response curve of assays of this type was not significantly different for the two types of injections. Only 2 dosage levels were used. The 2 dose assay rather than the 3 dose assay was adopted as a means of simplifying the necessary statistical calculations.[1]

Dosages of both standard and unknown are adjusted by dilution so that the expected response to all dosages will be between 1/20 and 19/20. This situation is not always fulfilled but at least 3 of the 4 points must be within these limits. The dilution factor is used in the calculation of potency and standard error of the assay, and is most conveniently expressed as

$$\frac{\text{I.U. of standard per cc.}}{\text{Assumed I.U. of unknown per cc.}}$$

In all cases 20 animals were used on each point.

[1] At an informal meeting of the Subcommittee on Estrogen Assays, U.S.P. Endocrine Products Advisory Board, the use of a 2 dose assay was suggested as a means of simplifying the interpretation of the assay results. This report is a direct outgrowth of that suggestion.

DESIGN OF THE ASSAY

The results of a typical assay are presented graphically in Figure 1. The observed points are plotted log doses of I.U. (or mg.) against response in degrees angle θ. The response is transformed from the fraction of animals responding (for instance, 11/20) to degrees of angle θ by reference to Table 1 wherein fraction responding $= \sin^2 \theta$. These points are designated as S_H and S_L for the high and low dose of the standard, and U_H and U_L for the high and low dose of the unknown.

The difference between the logarithms of the high and low doses is "i" and the value of "i" must be the same for both standard and unknown, in this case 0.1. (It follows that "i" is also the logarithm of the ratio of the two doses.) The slope of the line that would pass through the plotted points of the standard would be

$$\frac{S_H - S_L}{i}$$

and similarly the slope for the unknown would be

$$\frac{U_H - U_L}{i}.$$

The average slope for the two lines would be

$$\text{Slope} = b = \frac{(S_H - S_L) + (U_H - U_L)}{2i} = \frac{W}{2i}$$

where $W = (S_H - S_L) + (U_H - U_L)$. In the same way the vertical distance between the two lines would be

$$\text{Vertical distance} = \frac{(U_L - S_L) + (U_H - S_H)}{2} = \frac{V}{2}$$

where

$$V = (U_L - S_L) + (U_H - S_H).$$

M, the horizontal distance between the lines (see figure 1) measures the ability of the standard and unknown to produce the same response and hence is a measure of the potency. In terms of log dose, the potency would be

$$M = \log \text{potency} = \text{horizontal distance} = \frac{\text{vertical distance}}{\text{slope}} = \frac{iV}{W}.$$

METHODS OF ANALYSIS

1. *Evaluation using the graph and nomograph.* It is necessary to make only two simple computations in order to use the graph (figure 2) for determining potency, and two more to obtain the standard error of the assay using the nomograph (figure 3).[2]

[2] Enlarged graphs and nomographs are available upon request to the Division of Pharmacology, Food and Drug Administration, Washington 25, D. C.

TABLE I. TRANSFORMATION OF RESPONSE FROM FRACTIONS TO DEGREES OF ANGLE θ

Where fraction responding $= \sin^2 \theta$

(Modified from table XIII of Statistical Tables for Biological, Agricultural and Medical Research, R. A. Fisher and F. Yates, Oliver and Boyd, Ltd., Edinburgh, 1938, by permission of the authors and the publishers)

Number of Animals Responding	Total Number of Animals																								
	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
1	26.6	24.1	22.2	20.7	19.5	18.4	17.5	16.8	16.1	15.5	15.0	14.5	14.0	13.6	13.3	12.9	12.6	12.3	12.0	11.8	11.5	11.3	11.1	10.9	10.7	10.5
2	39.2	35.3	32.3	30.0	28.1	26.6	25.2	24.1	23.1	22.2	21.4	20.7	20.1	19.5	18.9	18.4	18.0	17.5	17.2	16.8	16.4	16.1	15.8	15.5	15.2	15.0
3	50.8	45.0	40.9	37.8	35.3	33.2	31.5	30.0	28.7	27.6	26.6	25.7	24.8	24.1	23.4	22.8	22.2	21.7	21.2	20.7	20.3	19.9	19.5	19.1	18.8	18.4
4	63.4	54.7	49.1	45.0	41.8	39.2	37.1	35.3	33.7	32.3	31.1	30.0	29.0	28.1	27.3	26.6	25.9	25.2	24.6	24.1	23.6	23.1	22.6	22.2	21.8	21.4
5	90.0	65.9	57.7	52.2	48.2	45.0	42.4	40.2	38.3	36.7	35.3	34.0	32.8	31.8	30.9	30.0	29.2	28.5	27.8	27.2	26.6	26.0	25.5	25.0	24.5	24.1
6		90.0	67.8	60.0	54.7	50.8	47.6	45.0	42.8	40.9	39.2	37.8	36.4	35.3	34.2	33.2	32.3	31.5	30.7	30.0	29.3	28.7	28.1	27.6	27.1	26.6
7			90.0	69.3	61.9	56.8	52.9	49.8	47.2	45.0	43.1	41.4	39.9	38.6	37.4	36.3	35.3	34.3	33.5	32.7	31.9	31.3	30.6	30.0	29.4	28.9
8				90.0	70.5	63.4	58.5	54.7	51.7	49.1	46.9	45.0	43.3	41.8	40.5	39.2	38.1	37.1	36.1	35.3	34.4	33.7	33.0	32.3	31.7	31.1
9					90.0	71.6	64.8	60.0	56.3	53.3	50.8	48.6	46.7	45.0	43.5	42.1	40.9	39.8	38.7	37.8	36.9	36.0	35.3	34.5	33.9	33.2
10						90.0	72.5	65.9	61.3	57.7	54.7	52.2	50.1	48.2	46.5	45.0	43.6	42.4	41.3	40.2	39.2	38.3	37.5	36.7	36.0	35.3
11							90.0	73.2	66.9	62.4	58.9	56.0	53.6	51.4	49.5	47.9	46.4	45.0	43.8	42.6	41.6	40.6	39.7	38.8	38.0	37.3
12								90.0	73.9	67.8	63.4	60.0	57.2	54.7	52.6	50.8	49.1	47.6	46.2	45.0	43.9	42.8	41.8	40.9	40.0	39.2
13									90.0	74.5	68.6	64.3	61.0	58.2	55.8	53.7	51.9	50.2	48.7	47.4	46.1	45.0	43.9	43.0	42.0	41.2
14										90.0	75.0	69.3	65.2	61.9	59.1	56.8	54.7	52.9	51.3	49.8	48.4	47.2	46.1	45.0	44.0	43.1
15											90.0	75.5	69.9	65.9	62.7	60.0	57.7	55.7	53.9	52.2	50.8	49.4	48.2	47.0	46.0	45.0
16												90.0	76.0	70.5	66.6	63.4	60.8	58.5	56.5	54.7	53.1	51.7	50.3	49.1	48.0	46.9
17													90.0	76.4	71.1	67.2	64.1	61.5	59.3	57.3	55.6	54.0	52.5	51.2	50.0	48.8
18														90.0	76.7	71.6	67.8	64.8	62.2	60.0	58.1	56.3	54.7	53.3	52.0	50.8
19															90.0	77.1	72.0	68.3	65.4	62.8	60.7	58.7	57.0	55.5	54.0	52.7
20																90.0	77.4	72.5	68.8	65.9	63.4	61.3	59.4	57.7	56.1	54.7
21																	90.0	77.7	72.8	69.3	66.4	64.0	61.9	60.0	58.3	56.8
22																		90.0	78.0	73.2	69.7	66.9	64.5	62.4	60.6	58.9
23																			90.0	78.2	73.6	70.1	67.4	65.0	62.9	61.1
24																				90.0	78.5	73.9	70.5	67.8	65.5	63.4
25																					90.0	78.7	74.2	70.9	68.2	65.9
26																						90.0	78.9	74.5	71.2	68.6
27																							90.0	79.1	74.8	71.6
28																								90.0	79.3	75.0
29																									90.0	79.5
30																										90.0

To determine the potency of an unknown calculate

$$V = U_H + U_L - S_H - S_L$$
$$W = U_H + S_H - U_L - S_L.$$

On the potency chart (figure 2) find the point corresponding to the calculated values of V and W and read the potency from the radial lines.

To determine the standard error of the assay calculate

$$Z = V^2 + W^2$$

using the partial table of squares in Figure 3. Find on Figure 3 the values of W and Z on their respective scales. Connect these two points with a straight

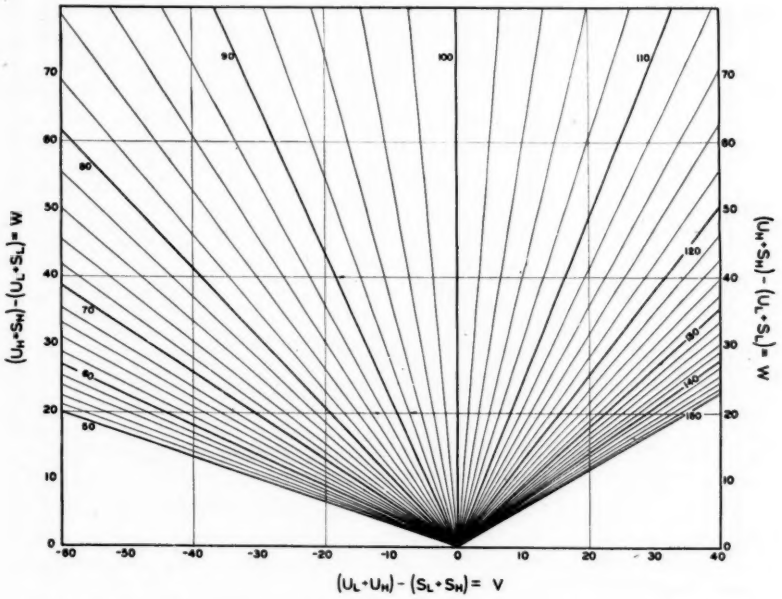

FIG. 2. Estrogenic assay—chart for determining potency as percent of standard from two-dose method. H = high dose, L = low dose, $\frac{H}{L} = 1.259$.

edge and read the value of the ratio at the point of intersection of the straight edge with the ratio scale. Multiply this value of the ratio by the previously found potency to obtain the standard error of the assay.

Ratio × Potency = Standard Error of the Assay

Both of these values are then corrected for the dilution factor described under Method of Assay by multiplying each by the dilution factor.

Example of the use of graph and nomograph on the results of a typical assay. From a knowledge of the expected potency of the unknown, the measurement of oil to be used in the dilution can be calculated so that the unknown solution will contain the same number of I.U. per cc. as the standard solution.

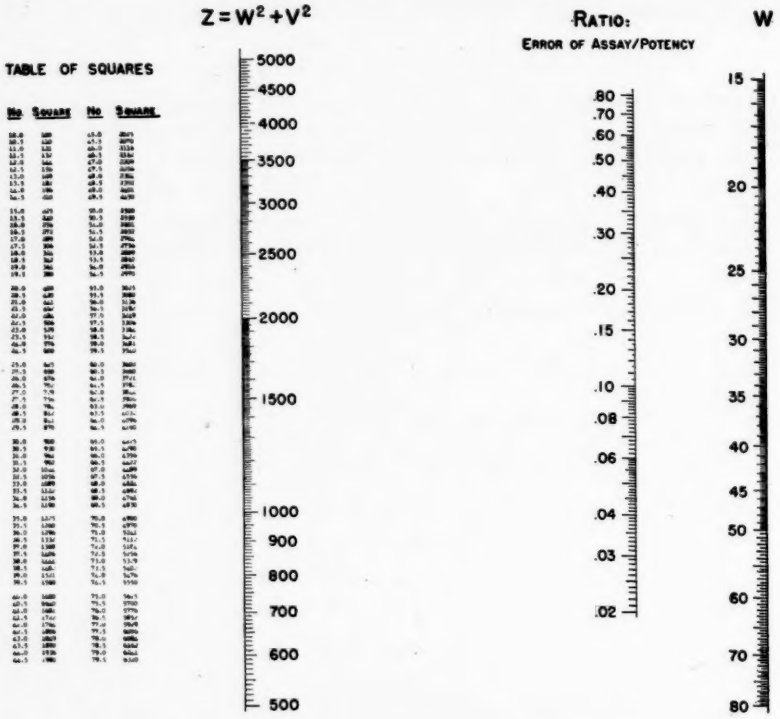

FIG. 3. Estrogenic assay—nomograph for determining standard error of the assay as percent of standard. For $i = 0.1$ and $N = 20$.

However, it is often inconvenient to measure volumes of oil to the third and fourth decimal places. For convenience the measurements are rounded to two decimal places and the concentration recalculated. The dilution factor for this assay is:

$$\text{Dilution factor} = \frac{\text{I.U. of standard/cc.}}{\text{Assumed I.U. of unknown/cc.}} = \frac{52}{56} = .928$$

	Dose, I.U.	*Log Dose*	*Response*	*Degrees, θ*
S_H	13.1	1.117	17/20	67.2
S_L	10.4	1.017	11/20	47.9
U_H	14.1	1.149	18/20	71.6
U_L	11.2	1.049	11/20	47.9

$$V = 71.6 + 47.9 - 67.2 - 47.9 = 4.4$$

$$W = 71.6 + 67.2 - 47.9 - 47.9 = 43.0$$

Potency from the graph = 102.4

$$Z = V^2 + W^2 = 19.36 + 1849.0 = 1868.36$$

Ratio from nomograph = .069.

Standard error = 102.4 × .069 = 7.1
Correction for dilution factor:

$$\text{Potency} = 102.4 \times .928 = 95.0$$

$$\text{Standard error} = 7.1 \times .928 = 6.6$$

$$\text{Potency} = 95.0 \pm 6.6\% \text{ of standard.}$$

2. *Evaluation without the graph and nomograph.* Upon occasions it is neither desirable nor convenient to prepare dilutions of the standard and unknown in such a way that $i = 0.1$ and at times assays will be conducted with some number of animals other than 20 on each point. In this case, as long as the number of animals (n) on each point is equal, the following calculations may be used for determining the potency of an unknown and the standard error of the assay. In the following example the unknown was assumed to have a potency equal to the standard and the dilutions so prepared. Hence the dilution factor is 1.

	Dose, I.U.	*Log Dose*	*Response*	*Degrees, θ*
S_H	22.4	1.350	21/26	64.0
S_L	14.1	1.149	7/26	31.3
U_H	22.4	1.350	16/26	51.7
U_L	14.1	1.149	5/26	26.0

$$V = U_H + U_L - S_H - S_L = -17.6 \qquad b = \frac{W}{2i} = 145.2$$

$$W = U_H + S_H - U_L - S_L = 58.4 \qquad \sigma_V^2 = \Sigma(1/4n) = .038460$$

$$i = 1.350 - 1.149 = .201 \qquad \sigma_V = .196112$$

$$M = \frac{iV}{W} = -.0606$$

$$\sqrt{Z} = \sqrt{V^2 + W^2} = \sqrt{3720.32} = 60.9944$$

$$d = \frac{i\sigma_V\sqrt{Z}}{W^2} = .000704$$

Potency = antilog $(2 + M)$ = antilog 1.9394 = 87.0

Standard error of the assay = 131.92 × potency × d = 8.1

Potency = 87.0 ± 8.1% of standard.

DISCUSSION

The analysis of a series of assays arranged to allow comparison of their interpretations by both the probit and the graphic method are given in Table 2. Each assay is a comparison of different solutions of U.S.P. Reference Standard Estrone wherein the "unknown" is just 80% of the "standard". In none of the eleven assays is there any significant difference between the evaluations of the same assay by the probit method and the graphic method, nor is any trend indicated that one method gives results consistently higher or lower than the

other. Also in Table 2 a series of 2 dose assays (No. 1-6) in which the total dose was administered in *two* equal injections is compared with another series of 2 dose assays (No. 7-11) in which the total dose was administered in *three* equal injections. From these limited data it appears that increasing the number of injections has no significant

TABLE 2. ASSAYS OF SOLUTIONS OF KNOWN POTENCY (80% OF STANDARD)

Assay No.	Method of Assay—2 dose, 2 injection					
	Probit interpretation			Graphic interpretation		
	Potency		Std. error of the assay	Potency		Std. error of the assay
1	82.7	±	11.2	83.4	±	10.6
2	91.8	±	7.8	91.9	±	7.9
3	69.8	±	25.1	69.5	±	24.1
4	62.7	±	16.9	62.3	±	17.0
5	95.6	±	10.9	94.5	±	11.8
6	83.4	±	9.7	84.1	±	8.4
Avg. potency	80.2			80.9		
Avg. std. error of assay			16.5			16.0
Std. deviation of potency	13.0			13.1		
	Method of Assay—2 dose, 3 injection					
Assay No.	Probit interpretation			Graphic interpretation		
7	78.6	±	8.8	78.7	±	7.6
8	96.1	±	5.4	96.2	±	4.7
9	69.2	±	27.9	71.4	±	24.6
10	73.1	±	15.7	70.8	±	11.7
11	85.3	±	7.8	86.2	±	7.6
Avg. potency	79.9			80.2		
Avg. std. error of assay			17.2			14.8
Std. deviation of potency	10.4			10.4		
	Method of Assay—3 dose, 3 injection					
Assay No.	Probit interpretation					
A	85.9	±	19.8			
B	89.7	±	6.8			

Note: The averages and standard deviations of the potency were obtained through the use of logarithms of the potencies. The average standard errors of the assay were obtained by the use of the squares of the individual standard errors of the assay.

effect on the average potencies found by the two methods. However, the standard deviations of the potencies found in these 2 series are slightly different. Statistically those for the 3 injection method, though smaller, are not significantly different from those for the 2 injection method (10.4 as compared to 13.0). If this apparent trend toward wider deviations becomes conclusive as a result of future accumulation of determinations it may become necessary to adopt the 3 injection, 2 dose technique as a means of reducing the variation

of the assay. The standard deviation of the potencies could be calculated since in all assays the true potency was 80%. Thus the standard deviation in this case measures the variation about the true potency. It should be noted that the average standard error in each case is greater than the standard deviation of the potency. This indicates that at least the standard error of the assay at a single laboratory is not being underestimated. Whether this would be true in a collaborative experiment involving several laboratories is open to question.

The results of the 3 dose, 3 injection assays are included in Table 2 for purposes of comparison with the results of the 2 dose method. On the basis of the values at hand there seems to be no reason to continue using the more complicated 3 dose method.

In reference to objections raised by assayists to the use of statistical methods of analysis, the time required to interpret the same assay by the two methods is of interest. The graphic method of analysis required 6 minutes while the probit method (Miller et al. 1939) required 1 hour and 10 minutes. This comparison of time required was made by a person very familiar with both techniques.

Finally it must be stressed that there are three conditions in the design of the assay which must be met before use of the graphic method of analysis is valid. 1) The number of animals on each point must be twenty. This requirement may be relaxed to the extent that any number of animals may be used provided an equal number of animals are used on each point if the calculations illustrated for the error of the assay are used instead of the nomograph. 2) The logarithmic interval between the high and low dose of both standard and unknown must be 0.1. Again this requirement may be liberalized to the extent that any reasonable logarithmic interval may be used but unknown and standard must have the same interval, and the calculations for potency and error of the assay must be used instead of the graph and nomograph. (A reasonable logarithmic interval is one that is smaller than the linear range of the dosage-response curve.) 3) The value of W must be greater than 20. This requirement simply assures that the slope of the dosage-response line differs significantly from zero. As a corollary to this requirement it may be pointed out that in the mathematical development of the graphic method of analysis it was assumed that the slope of the dosage-response lines of standard and unknown were not statistically different. In other words, given the same percentage increase in the doses of standard and unknown, the amount of increase in the responses (in terms of degrees θ) will be the same. Thus when a comparison is desired of the estrogenic potency of two compounds which are known to have different slopes of their dosage-response lines, such as estrone and stilbestrol, neither the graphic nor computation method of analysis presented herein is valid. The experience in this laboratory agrees with that of Emmens (1939) in that the slopes of the dosage-response lines of estrone, estradiol, and im-

pure extracts from natural sources do not differ significantly from each other. Information on the slope of the dosage-response line on synthetic estrogens other than stilbestrol is not available.

In this paper, the standard error of the assay measures the variation in potencies caused by the factors that vary *within* any one assay. This standard error of the assay is to be contrasted with the standard error of the potency which measures the variation in potencies caused by factors that vary between assayists and between laboratories. These two standard errors may be regarded respectively as the error within an assay, and the error between assays run at different times at different laboratories. They cannot be considered equal until they have been shown to be equal by means of one or more collaborative assays.

SUMMARY

A graph and nomograph have been prepared for the statistical evaluation of the results of estrogenic assays. The use of these charts requires only 4 simple calculations and the results expressed in terms of potency as percent of standard and the standard error of the assay in the same terms can be obtained in about 6 minutes. The use of the graph and nomograph depend upon the strict adherence to a special design of the assay described and the transformation of response to an angular function.

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THE IMPOSITION OF DIURNAL[1] OVULATORY AND TEMPERATURE RHYTHMS BY PERIODIC FEEDING OF HENS MAINTAINED UNDER CONTINUOUS LIGHT[2]

R. M. FRAPS, B. H. NEHER, AND I. ROTHCHILD
From the Agricultural Research Center
BELTSVILLE, MARYLAND

IN CONNECTION with experiments on the effect of light on time of ovulation of the first follicle of the hen's clutch,[3] we had occasion to maintain birds under continuous artificial lighting, with ample feed and water available at all times and in quarters from which natural daylight was completely excluded. The hens received fresh feed and water, and the usual maintenance operations were carried out between 8:00 A.M. and 4:00 P.M. The birds were in two completely separated rooms, but were subjected to outside noises and disturbances, such as crowing cocks in the early morning, traffic disturbances, and operation of the elevator over a considerable part of the day. After having been maintained under these conditions for as long as four weeks the hens continued to lay a very large proportion of all eggs during the 12 hour period, 6:00 A.M.–6:00 P.M., and during the last week of continuous and uniform lighting all eggs were laid between 6:00 A.M. and 8:00 P.M. (Group I, Table 1). These hours, 6:00 A.M.–8:00 P.M., were the same as those during which the hens had been maintained under artificial illumination for several months previous to the initiation of continuous lighting.

Since the hens in question were subjected to continuous and uniform lighting, the restriction of oviposition within about 12 or 14 hours of the 24 hour day must obviously have been regulated by some stimulus other than any diurnally recurrent alternation of light and darkness, i.e., photoperiodicity as such. The behavior of our hens was

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[1] Welsh (1938) has called attention to the ambiguity attaching to the term "diurnal." In view of the common application of the notation to indicate rhythmic variations within a 24 hour cycle, we shall use it in the present paper in accordance with this accepted practice.

[2] An abstract of the subject matter of this paper has previously been published (Fraps, 1946).

[3] The clutch is defined as the number of eggs laid on successive days, separated from other clutches by one or more days during which no eggs are laid. We shall use the term in the present paper to designate also the sequence of ovulations occurring on successive days; as Warren and Scott (1936) point out, this sequence of ovulations begins in the early morning and as a rule is terminated at around 12:00 noon to 2:00 P.M. under natural lighting conditions.

also in substantial disagreement with the observations of Warren and Scott (1936) and McNally (1946),[4] who reported that battery caged hens maintained under continuous illumination laid their eggs, following the few days required for adjustment to the novel condition, at any time of the day, approaching a random distribution over the 24 hours of the astronomical day.

The results recorded by Warren and Scott (1936) for battery caged hens are of particular interest in this connection, since these authors state that feed and exercise seemed not to be factors influencing the time of oviposition. They noted also, however, that one group of hens with freedom of the floor space of the laying house failed to lay at random under continuous lighting, possibly because of a small leakage of outside light, of avoidance of direct rays of the artificial light, or because of psychological factors. These birds continued to lay approximately as though they were on a natural diurnal-nocturnal rhythm, corresponding presumably to a 12 to 14 hour light day. Warren and Scott also called attention to the fact that birds subjected to extended artificial lighting as a regular commercial practice laid their eggs within a limited portion of the natural light-day, not at random during the 24 hours. They appear to have concluded that the superimposed effect of artificial lighting was not a sufficient stimulus to break up the rhythm based upon the period of natural daylight.

The observations on times of lay reported in this paper were designed mainly to determine whether periodic feeding, and the concomitant restriction of activity in laying hens maintained under continuous illumination, would also limit the time of oviposition in a manner comparable to the effect of photoperiodicities.

In view of the failure of the birds to come into random lay under continuous light, it was also of interest to determine whether variations in body temperatures were comparable with temperature cycles exhibited by birds maintained under 24-hour photoperiodicities, a point of considerable theoretical significance in view of the more or less implicit assumption by some previous workers that photoperiodicities determine this rhythm in birds. Hildén and Stenbäck (1916) and Huff (1939) have shown that the diurnal-nocturnal rhythm in body temperature is readily reversed under reversed light-dark sequences. Simpson and Galbraith (1905) recorded a reversal of the usual diurnal-nocturnal temperature rhythm in birds active during night hours, an observation confirmed by Wetmore (1921).

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[4] It was not known, at the time the experiments recorded here were undertaken, that McNally had carried out work along similar lines several years ago in connection with other investigations at the University of Maryland. McNally's findings have recently been published (1946). The results reported in the present communication confirm McNally's results on the association of the feeding schedule with times of oviposition.

MATERIALS AND PROCEDURES

Approximately equal numbers of White Leghorn and Rhode Island Red hens, most of which were well into their second year of production at the time the experiments were begun, served as experimental material. All birds were individually caged in laying batteries, with feed and water available throughout the period indicated in the several experiments (Table 1). The number of laying hens under observation varied considerably in different tests in consequence of birds going out of production or coming into production. Two of the batteries, each with a capacity of 48 hens, were in two rooms separated by a partition but with an opened doorway between them. The third battery, with a capacity of 60 hens, was in a room separated from any other part of the building by two doors with a vestibule between them.

TABLE 1. RELATION BETWEEN FEEDING SCHEDULES AND OVIPOSITION BY HENS MAINTAINED UNDER CONTINUOUS LIGHT (EXCEPTING GROUP DESIGNATED AS CONTROLS)

Test number	Feeding schedule	Days of record	All eggs laid	Active period[a]		Eggs laid, active period	
			no.	*times*	*hrs.*	*no.*	*%*
Control[c]	Continuous[b]	1–10 (10)	339	6 A.M.–6 P.M.	12	334	98.5
I	Continuous	1–10 (10)	301	6 A.M.–6 P.M.	12	266	88.4
	Continuous	1–10 (10)	301	6 A.M.–8 P.M.	14	294	97.7
	Continuous	11–20 (10)	291	6 A.M.–6 P.M.	12	234	80.4
	Continuous	11–20 (10)	291	6 A.M.–8 P.M.	14	289	99.3
	Continuous	21–28 (8)	274	6 A.M.–6 P.M.	12	243	88.4
	Continuous	21–28 (8)	274	6 A.M.–8 P.M.	14	274	100.0
II	8 A.M.–4 P.M.	1–18 (18)	202	6 A.M.–6 P.M.	12	192	95.0
	8 A.M.–4 P.M.	1–18 (18)	202	6 A.M.–8 P.M.	14	197	97.5
III	8 P.M.–4 A.M.	10–19 (10)	433	6 P.M.–6 A.M.	12	311	71.8
	8 P.M.–4 A.M.	10–19 (10)	433	6 P.M.–8 A.M.	14	323	76.4
	8 P.M.–4 A.M.	10–19 (10)	433	4 P.M.–6 A.M.	14	369	85.2
	8 P.M.–4 A.M.	10–19 (10)	433	4 P.M.–8 A.M.	16	381	88.0
IV	8 A.M.–4 P.M.	7–15 (9)	323	6 A.M.–6 P.M.	12	289	89.5
	8 A.M.–4 P.M.	7–15 (9)	323	6 A.M.–8 P.M.	14	292	90.4

[a] This period covers the main term of observation, some records, however, having been taken over somewhat longer periods.

[b] The control hens were maintained under lights from 6:00 A.M.–8:00 P.M. for several months before initiation of the experiments, feed and water available throughout the 24 hours; the indicated 10 day of record is for the 10 days preceding the tests under continuous lighting.

Illumination in all rooms was provided by 60 watt lamps mounted 7 feet from the floor and about 30 inches apart, directly in front of the batteries. In the double room referred to above the purpose of the partition was to carry these lamps; in these rooms the batteries were about 3 feet from the walls upon which the lamps were mounted, and the cages in all tiers of the batteries were well illuminated. In the other room the lights were somewhat further from the batteries, but ample illumination was provided to all hens in all tiers of the battery.

There seems little possibility that the behavior of birds in one room may have influenced activity or any other cyclic phenomena of hens in the other rooms. The distribution in times of lay within the 24 hour cycle was similar in the two groups, and the only reasonable conclusion is that both groups of hens were responding to the same stimuli when the laying cycle was maintained independently of continuous lighting.

The hens were fed a standard laying mash, which was subject to some changes during the course of the experiments. Since we were not interested in rate of production as such, these changes were probably of little signifi-

cance. However, when feed was replenished, the hens tended to resume feeding almost as though they had not been fed for some hours; apparently the cracked grain in the mash was always an incentive in this reaction. The time of feeding may therefore have been a factor in periodically increasing activity of the birds, even though in some schedules (Group I of Table 1) all hens had feed before them at all times.

As was noted earlier, external disturbances and maintenance conditions may have been a factor in accounting for the results recorded below. These should probably be considered as experimental conditions to which the birds were subjected, although their control and evaluation are admittedly difficult. It can certainly be said that the hours from about 5:00 P.M. to around 4:00 A.M. were much more quiet than were hours during the remaining part of the day. While these factors may not have influenced the results materially, we cannot exclude the possibility that they contributed in part to the quantitative values recorded below.

RESULTS

The various feeding schedules employed and their association with times of lay are recorded in Table 1. The control group of birds was maintained under a 14 hour light schedule, lights from 6:00 A.M. through 8:00 P.M. Feed was available to these birds at all times, although replenished at approximately the same time daily. Over 98 per cent of all eggs laid during the 10 days preceding the initiation of the experiment under continuous light were laid between 6:00 A.M. and 6:00 P.M.

When the hens were first placed under continuous lighting it was expected that they would gradually tend to lay at random during the 24 hour period. Time of lay was accordingly recorded from 8:00 A.M. through 4:00 P.M. and again at 6:00 and 8:00 P.M. in order to estimate the shift from the previous laying schedule. It is clear from the data of Table 1, Group I, that even after 4 weeks the hens under continuous lighting and with feed available at all times exhibited no more than a 2 hour lag in time of lay when compared with the control group. The random distribution in times of lay described by Warren and Scott (1936) and confirmed by McNally (1946) was certainly not observed over this period of time. It is notable that the percentage of eggs laid within the 14 hour period, 6:00 A.M.–8:00 P.M., tends to increase rather than decrease during the 28 days the birds were maintained under continuous illumination, with feed available at all times.

At the conclusion of the foregoing observations, time of lay was recorded for the same hens at 3 hour intervals during 4 days. The total number of eggs laid was 117. Ninety-one, or 77.7 per cent, were laid between 6:00 A.M. and 6:00 P.M., and 94, or only 80.3 per cent, between 6:00 A.M. and 9:00 P.M. It seemed reasonably certain that some element of disturbance at regular intervals over the 24 hour day might alter the distribution in time of lay recorded for Test Group I.

At the end of this 4-day period, eggs were collected at 6:00 A.M.,

hourly from 8:00 A.M. through 4:00 P.M. and at 6:00 and 8:00 P.M. for 5 days. The total number of eggs laid was 93; of these 85, or 91.4 per cent, were laid between 6:00 A.M. and 8:00 P.M., in good accord with the results tabulated in Table 1 for birds during the first 28 days under constant illumination, feed available at all times.

The results of the above tests, while not conclusive, indicated that stimuli other than light might act to maintain the same rhythm in egg laying under continuous light and feeding as was obtained in the control group, i.e., under controlled photoperiodicities. The apparently significant change in distribution of times of lay occasioned by collecting eggs at 3 hour intervals suggested very strongly that some phase of the bird's activity was associated with time of lay, and therefore of ovulation. In order to test this supposition, all the hens maintained under continuous lighting, were allowed feed only from 8:00 A.M. through 4:00 P.M. At 4:00 P.M. the hoppers were removed and replaced the following morning at 8:00 A.M. The results recorded in Table 1, Group II, are practically identical with the distribution in times of lay for Group I, for the 6:00 A.M.–8:00 P.M. interval, but indicate a somewhat higher percentage of eggs laid between 6:00 A.M. and 6:00 P.M.

After 18 days on the 8:00 A.M.–4:00 P.M. feeding schedule, this schedule was exactly reversed, the hens receiving feed only between 8:00 P.M. and 4:00 A.M. Nine days were allowed for the hens to accommodate to the new schedule. The distribution in times of lay for the following 10 days are recorded in Table 1, Test Number III. Approximately 72 per cent of all eggs were laid during the 12 hour period, 6:00 P.M.–6:00 A.M., compared with only 5 per cent laid during the same period when the hens were fed from 8:00 A.M. through 4:00 P.M. About 76 per cent of all eggs were laid between 6:00 P.M. and 8:00 A.M., while only 2.5 per cent were laid between 8:00 P.M. and 6:00 A.M. when the hens had access to feed between 8:00 A.M. and 4:00 P.M.

In Experiment I and II most of the eggs not laid between 6:00 A.M. and 6:00 P.M. were laid between 6:00 P.M. and 8:00 P.M. In birds fed from 8:00 P.M.–4:00 A.M., more eggs were laid during the hours preceding the time the birds received feed than during similar periods following the removal of feed. The data bearing on this point are tabulated in Table 1 in the comparison between the number of eggs laid from 6:00 P.M.–8:00 A.M. and 4:00 P.M.–6:00 A.M.

After the hens had been maintained on Schedule III for 19 days, feed was again made available from 8:00 A.M. through 4:00 P.M., Test Number IV of Table 1. The distribution in time of lay of eggs was completely reversed by the 7th day. Records of lay are given in Table 1 for 9 days, beginning with day 7 following reversal of the feeding schedule. The percentage of eggs laid between 6:00 A.M. and 6:00 P.M. did not differ appreciably from the percentage laid between 6:00 A.M. and 8:00 P.M., a result somewhat in contrast with the data recorded

above for Schedule I. The proportion of eggs laid during the 6:00 A.M.–6:00 P.M. period is however sufficiently high to indicate that the great majority of hens were laying in conformity with the altered schedule of feeding.

Diurnal rhythms in body temperatures under restricted feeding. A group of 12 hens, 6 White Leghorns and 6 Rhode Island Reds, was used at the conclusion of Experiment III for the determination of body temperature. Body temperatures were taken for the same 12 hens at the conclusion of feeding Schedule IV, which was the exact reverse of Schedule III. In both cases rectal temperatures were obtained at 2 hour intervals over a period of 48 hours. The temperature curves, plotted with reference to feeding schedules rather than astro-

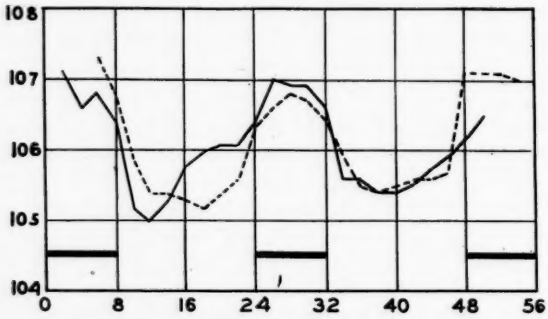

FIG. 1. Curves of rectal temperature of 12 hens taken at 2-hour intervals. Solid line, when fed between 8:00 A.M. and 4:00 P.M.; broken line, temperature curve for some hens when fed between 8:00 P.M. and 4:00 A.M. Black horizontal bars, feeding periods.

nomical time, are shown in Figure 1. The solid line represents body temperatures of hens having access to feed between 8:00 A.M. and 4:00 P.M., the broken line, for the hens feeding between 8:00 P.M. and 4:00 A.M. There are minor dissimilarities between the curves, but the alternation of maximal and minimal values indicates clearly that feeding, or the activity associated with feeding, is essentially the same for hens under both feeding schedules. The curves are altogether comparable with those recorded for hens maintained under recurrent photoperiodicities, the period of feeding of Figure 1 corresponding to the period of lighting for hens maintained under alternations of light and dark.

DISCUSSION

The experiments recorded here were based upon a single group of hens subjected to successive tests.

One of the most interesting aspects of the data recorded in this paper is that hens maintained under continuous illumination, with ample feed and water available at all times, failed to deviate signifi-

cantly in their laying behavior from that maintained by the same hens under a 14 hour light-day (controls, Table 1). The fact that these same hens continued to maintain practically the same rhythm in egg laying as they had under a 14 hour light-day might be taken as evidence that a basic physiological rhythm had been established under the conditions of the previous lighting schedule (lights from 6:00 A.M. through 8:00 P.M.). The reversal of the laying cycle in most hens under the conditions of Test Group III (feeding and servicing between 8:00 P.M. and 4:00 A.M.) would indicate, however, that such a rhythm was not inherent, nor was it permanently established. The complete reversal of the cycle of diurnal variation in body temperatures with reference to time of feeding and associated activity enforces this conclusion. It is to be emphasized again that all the experiments recorded in this paper were carried out under continuous and uniform illumination, and there was accordingly no possibility that photoperiodicity conditioned any of the diurnally recurrent phenomena.

Two hypotheses have been formulated with respect to the causal factors effecting seasonal gonadal development in birds. Rowan (1928, 1937, and 1938) believes the bird's activity to be the primary stimulus in gonadal development. Bissonnette (1931, 1936), Burger, Bissonnette and Doolittle (1942), Riley (1937, 1940a), and Benoit (1935, 1937) have on the contrary considered light *per se* as the causal agency of the seasonal sexual cylce. Wolfson (1941) has attempted to reconcile these contrasting hypotheses by suggesting that increased daily illumination causes recrudescence of the gonads under both experimental and natural conditions because the birds are awake, and presumably active, for longer periods of time under longer lighting schedules. Our results demonstrate that alternating periods of activity and rest can be maintained independently of recurrent light and darkness. The environmental factors underlying seasonal gonadal development and regression may however be of a different order, but the procedures described in this paper might prove of value in resolving some phases of the problem.

The question arises as to whether time of feeding, and the intake of feed *per se*, controls the diurnal periodicities described in this paper. If one may assume a fairly direct correlation between variations in body temperature and oxidative metabolism, the determinations of oxygen consumption carried out by Barott, et al. (1938) may be taken to indicate that the diurnal temperature rhythms are not necessarily associated with the feeding schedule. The continuous curve representing oxygen consumption was established for fasting male chickens, age 4–130 days, over a period of 72 hours. The birds were kept in complete darkness, but the diurnal variation in oxygen consumption was exhibited throughout this period, although at lower absolute values during successive 24 hour intervals. The diurnal rhythm in oxygen consumption was obviously independent of any feeding schedule in these experiments.

Fraps, et al. (1942) found that intravenous injections of ovulation inducing hormones forced ovulation at a minimum of about 6 hours. Rothchild (1946) estimates the time between release of the ovulation-inducing hormone and ovulation at about 5 hours, basing his calculations upon results following hypophysectomies. It would appear therefore that the ovulation-inducing hormone is released at or near the time of minimal body temperature (Figure 1). This relationship is not necessarily causal, but it is of interest to note that spermatogensis proceeds at a more rapid rate during the period of minimal body temperatures (Riley, 1937, 1940b).

The cyclic variation in body temperatures associated with periodic feeding under continuous lighting is very similar to the temperature rhythms associated with photoperiodicities if the approximate interval during which feed is available be taken as the equivalent of a comparable lighted period within a 24 hour cycle. In both cases oviposition of the first egg of the clutch occurs near the end of the ascending limb of the curve representing body temperatures. If the same relationship between ovulation and oviposition holds for birds under periodic feeding and alternations in light and darkness, ovulation of the first egg of the clutch must occur near the end of the phase of increasing body temperatures.

A distinction should be made between the effect of these environmental factors which require prolonged action to effect a given result, such as gonadal development, and those factors which are normally subject to diurnal variation, as for example, the limited action of the anterior pituitary hormone in effecting ovulation. The results of our experiments may therefore have little bearing upon those noted above.

In connection with the role of photoperiodicity as against that of diurnal variables in regulating reproductive processes, it should be remembered that the anterior pituitary gland secretes two hormones, follicle stimulating (FSH) and luteinizing (LH). Ovulation in the hen is readily induced by LH preparations, but not by FSH preparations of anterior pituitary origin (Fraps, et al., 1943). Gonadal development on the contrary, is generally assumed to be predominantly dependent upon FSH. The results reported in this paper are based largely upon the action of diurnally recurrent variables, not with the long term effects associated with gonadal development. Some results bearing on this problem have been published by Fiske (1941).

The rhythms of temperature variation over the 24-hour day is not dependent on gonadal activity, nor presumably upon thyroid activity, since hypophysectomized as well as thyroidectomized hens maintained under a 14-hour light-day, exhibit a curve similar to that shown by normal birds (unpublished data from this Laboratory).

Two other observations of interest in connection with the data presented in this paper may be noted briefly.

Byerly and Moore (1941) found that a 26 hour day, 14 hours of light alternating with 12 hours of darkness, lengthened clutches by "assuring a lighted period of sufficient length to maintain pituitary and consequently ovarian activity shortly after ovulation," and they suggested further that their results were obtained "by permitting the birds adequate rest periods and thus delaying the onset of refractoriness." The conclusions of Byerly and Moore, as thus stated, would indicate that activity rather than photoperiodicity was the important factor in determining the ovulatory schedule of their birds.

In a recent paper Schneider (1946) reported that the inclusion of exercise drums in the breeding cages of wild house mice was necessary to bring the animals into estrus and consequent breeding. While this may appear to be an extreme case of the role of activity in connection with reproductive cycles, it may also be merely another aspect of the same factors which determine clutch structure in the hen.

SUMMARY

Battery caged hens, having been maintained under a 14-hour light-day for several months, were placed under continuous illumination, with feed and water always available, in order to obtain the random distribution in times of lay reported by previous investigators. The hens unexpectedly continued to lay out their clutches as they did under the 14-hour light-day. Experiments were accordingly undertaken to determine the extent to which enforced activity may have accounted for the results.

The hens were subsequently allowed feed only from 8:00 A.M. through 4:00 P.M. under continuous lighting. Most eggs were laid between 6:00 A.M. and 6:00 P.M. The body temperatures of 12 hens were taken at 2-hour intervals at the conclusion of this test. The cycle of temperature changes was similar to the curve obtained for hens subjected to a similarly limited period of lighting.

The same hens, constantly under uniform illumination, were then allowed feed only between 8:00 P.M. and 4:00 A.M. Within about 10 days the periodicity of both lay and body temperatures was reversed, bearing the same relation to the period of feeding as in the earlier tests.

It is concluded that photoperiodicity is not a necessary factor in the regulation of time of lay nor of the diurnal temperature cycle. The restriction of ovulation within a limited portion of the normally lighted day may accordingly be associated mainly with those factors which determine bodily activity rather than with photoperiodicities.

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THE EFFECT OF PREGNANCY COMPLICATED BY ALLOXAN DIABETES ON THE FETUSES OF DOGS, RABBITS AND RATS

HERBERT C. MILLER, M.D.

From the Department of Pediatrics, University of Kansas, School of Medicine and from the Department of Pediatrics, Yale University School of Medicine,

KANSAS CITY, KANSAS

THE following experiments were undertaken on dogs, rats and rabbits to determine whether or not it was possible to reproduce in animals some of the many striking phenomena that have been observed in the human fetus of the diabetic mother. It is well established that the fetal and neonatal mortality is increased among infants born to diabetic mothers and that there is a marked tendency for the human fetus to have an increased birth weight without evidence of a prolonged gestation period. Changes in the viscera and endocrine glands of some infants born to diabetic mothers have also been reported. Among these changes there has been described cardiac enlargement (Miller and Wilson, 1943), an increased glycogen content of the myocardium (Hurwitz and Irving, 1937) (Miller, Johnson and Durlacher, 1944) an increased erythropoiesis in the liver and other tissues (Miller et al., 1944), an increased eosinophilia in the anterior hypophysis (Schretter and Nevinny, 1930) (Bauer and Royster, 1937) (Okkels and Brandstrup, 1938) (Miller, 1945a), hyperplasia of the adrenal glands (Miller et al, 1944), hyperplasia of islands of Langerhans (Miller and Wilson, 1943) (Dubreuil and Anderodias, 1920) (Helwig, 1940) and hyperplasia of the gonads (Smyth and Olney, 1938) (Beuner, 1941).

Since the high mortality rate and the marked tendency towards an increased birth weight are the two outstanding characteristics among infants born to diabetic mothers, information on these two characteristics was primarily sought in the animal experiments to be reported.

METHODS

Diabetes Mellitus was induced in all female animals by the injection of alloxan. Animals failing to develop significant glycosuria following the injection of alloxan were excluded from this study. All animals were kept in suitable metabolism cages and the urine was collected over a period of 24

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hours. The amount of sugar in the urine in each 24-hour period was determined by Somogyi's method (Somogyi, 1941). Attempts were made to collect urine every day throughout all the diabetic pregnancies and for the most part this was done, but not with complete success, chiefly because collection was interrupted in some instances while the animals were being bred and during hot weather when breeding was unlikely to occur.

All rats and rabbits were bred after becoming diabetic. The dogs were bred 19 to 23 days before alloxan was injected. In about half of all the diabetic pregnancies the gestation period was accurately determined. In the dogs and rabbits the time of coitus was observed and in the rats the typical

TABLE 1. AMOUNT OF GLYCOSURIA PER DAY IN 9 DIABETIC RABBITS WITH AMOUNT OF INSULIN INJECTED PER DAY

Rabbit number	Period of diabetes (days)	Number of daily urines	Average glycosuria per day (grams)	Range of glycosuria per day (grams)	Average insulin per day (units)	Range of insulin per day (units)
II	307	213	8	0–25	2	0–6
VI	272	175	13	1–47	3	0–8
VIII	107	80	10	2–22	2½	0–6
IX	158	96	8	1–24	2½	0–5
XI	35	35	5	0–20	2½	0–6
XIII	260	252	6½	1–22	3	0–8
XIV	126	91	5½	0–15	2	0–5
XVI	41	38	1	0–5	0	0
XIX	65	65	2¼	0–15	0	0

vaginal plug or the presence of sperm on microscopic examinations of smears taken from the vagina were noted. The day of coitus was counted as the first day of the gestation period. The Yale strain of albino rat and New Zealand White rabbits, both large and small varieties, were used in the experimntal and control groups. The same males served the control and diabetic groups of female rats and rabbits respectively.

Protamine zinc insulin was administered to the diabetic animals whenever it was deemed necessary. No attempt was made to render the urine sugar free, but only to prevent severe glycosuria.

RESULTS

Nine diabetic rabbits were followed through 17 pregnancies. The data concerning their diabetes are summarized in Table 1. For the most part the glycosuria was more or less constant in each rabbit, but in some animals there were periods of several days during which the glycosuria ceased or almost disappeared only to recur again. These remissions in the diabetic animals could not be accounted for on the basis of insulin received, since the latter was stopped during these periods.

The data on the 17 pregnancies in the 9 diabetic rabbits are given in Table 2. There were only two of the eleven pregnancies among the large New Zealand White rabbits that can be considered as full term pregnancies, numbers 10 and 11. Four pregnancies, 1 - 4, ended in

abortion with reabsorption of the fetuses as denoted by the appearance of a copious bloody vaginal discharge on the 17 day in one pregnancy, number 1, and on the 25 day in two other pregnancies, numbers 2 and 3. The day of pregnancy on which the abortion occurred could not be determined in the fourth pregnancy, number 4. The abortion in rabbit XI is noteworthy because during the week

TABLE 2. RESULTS OF 17 PREGNANCIES IN 9 DIABETIC RABBITS

	Pregnancy number	Rabbit number	Result	Litter size	Found dead	Average birth weight (grams)	Length of pregnancy (days)
Large New Zealand White	1	II	Abortion	—	—	—	17
	2	IX	Abortion	—	—	—	25
	3	XI	Abortion	—	—	—	25
	4	VI	Abortion	—	—	—	N.D.
	5	IX	Premature Delivery	2	2	N.D.	21
	6	II	Premature Delivery	7	7	11.0	N.D.
	7	XVI	Stillbirth (Macerated)	8	8	14.0	29
	8	IX	?Immature	6	1	32.8	N.D.
	9	IX	?Immature	7	5	22.2	N.D.
	10	XIX	Term	9	0	52.5	33
	11	XIX	Term	11	11	40.0	30
Small New Zealand White	12	VIII	Abortion	—	—	—	N.D.
	13	XIII	?Immature	6	0	29.1	N.D.
	14	XIII	Term	6	0	35.6	34
	15	XIII	Term	5	0	52.0	34
	16	XIII	Term	5	0	58.7	33
	17	XIV	Term	3	0	38.0	N.D.
	Total			75	34		

N.D.—Not Determined.

prior to the termination of this pregnancy there were only two days on which there was any glycosuria and on these two days there were only 5 and 3 grams of sugar excreted in the urine respectively. Two pregnancies, numbers 5 and 6, resulted in premature deliveries, one on the 21 day of pregnancy, number 5, and the other on an undetermined day. The very definitely reduced birth weight of the seven fetuses in number 6 pregnancy, which averaged only 11 grams each, indicates that this was a premature delivery. One pregnancy, number 7, resulted in the birth of three macerated fetuses, the largest of which weighed 20 grams, on the 29 day of the pregnancy. This rabbit, number XVI delivered a fourth macerated fetus on the 31 day and died three days later, at which time four more macerated fetuses averaging 9 grams apiece were found remaining in the uterus. This stillbirth occurred in a mildly diabetic rabbit. During the last three

weeks of the pregnancy not more than one or two grams of sugar was being excreted each day and the fasting blood sugar on the day of death was 167 mgm. per cent. Two pregnancies, numbers 8 and 9, ended in the delivery of fetuses the average birth weight of which was only about half of fetuses born after normal pregnancies, Table 3. The gestation periods could not be determined in the latter two pregnancies, so that it is not known whether or not the low birth weights of the fetuses were the result of premature deliveries or a failure to gain adequately during a normal gestation period. Among the small New Zealand White rabbits there was one abortion, pregnancy 12, and one questionably immature pregnancy, number 13.

TABLE 3

Results of Pregnancies in 7 large normal New Zealand White Rabbits

Rabbit number	Litter size	Found dead	Average birth weight (grams)	Length of pregnancy (days)
XVIII	2	2 (1 eaten)	76	27
XXI	2	0	78.5	35
XVI	4	2 (2 eaten)	44.0	33
XVII	6	1 (1 eaten)	58.0	34
XIX	9	0	58.0	33
XXII	9	0	61.4	33
XX	9	4 (1 eaten)	46.1	33
Total	41	9 (5 eaten)		

Three of the term deliveries, pregnancies 14, 15 and 16 were attributed to one rabbit, number XIII. The diabetes in this rabbit was more severe than in some of the rabbits that had less successful pregnancies, such as rabbits XI and XVI.

In none of the diabetic rabbits was there any evidence that the birth weight of the fetuses was greater than among normal rabbits. The average birth weights of seven litters born to large New Zealand White rabbits after normal pregnancies are given in Table 3. Two of the normal rabbits listed in Table 3, number XVI and XIX, had litters before being injected with alloxan and also after becoming diabetic. Rabbit XIX had two normal litters at term after the injection of alloxan had produced a mild diabetes. Rabbit XIV had a term delivery before receiving alloxan and after the diabetes was induced delivered 8 macerated fetuses.

The over-all mortality among the rabbit fetuses was definitely worse in the diabetic group, in which 34 of the 75 fetuses were found dead, as against 9 of the 41 fetuses born to normal rabbits. Half of the mortality among the normal rabbits was the result of the fetuses being eaten thus making the differences in the mortality figures between the experimental and control group more striking. The cause

of death in the experimental group of rabbits was largely caused by prematurity and stillbirth.

RATS

Significant glycosuria developed in 8 rats and they were followed

TABLE 4. GLYCOSURIA IN 8 DIABETIC RATS AND TREATMENT WITH INSULIN

Rat number	Period of[1] collection (days)	Average glycosuria per day (grams)	Range of glycosuria per day (grams)	Average insulin per day (units)	Range of insulin per day (units)
1	19	.66	.1 –2.2	1	0–5
1	8	1.6	.1 –4.5	3	0–5
2	10	.48	.1 –1.5	0	0
5	9	1.14	1.0 –2.0	2	0–4
6	19	.27	.1 –1.5	0	0
7	13	.5	0 –1.5	0	0
8	16	.35	.1 –2.0	2	1–3
9	3	.3	.1 – .4	1½	1–2
9	5	.72	.06–1.5	1	1–2
10	16	.13	.04–1.02	1	0–2

[1] Refers only to number of days in each pregnancy during which urine was collected.

TABLE 5. AVERAGE BIRTH WEIGHT OF LITTERS BORN TO NORMAL AND DIABETIC RATS

Number of rats in each litter	5	6	7	8	9	10	11
	Normals						
Number of litters	9	10	18	13	19	18	20
Weight of litter (gm.) Range low	5.3	4.5	4.8	4.6	4.5	4.7	4.6
Weight of litter (gm.) Range high	6.4	6.5	6.1	5.9	5.8	6.5	5.8
	Diabetic						
Number of litters	2	3	1	1	2	0	1
Weight of each litter (gm.)	4.7 5.8	4.5 6.0 6.3	5.7	4.5	5.4 5.8		6.4

through 10 diabetic pregnancies. The data relevant to the diabetes in these 8 rats are given in Table 4. Glycosuria was not considered significant unless there was one per cent or more sugar in the urine at some time during each pregnancy. The length of the gestation period was accurately determined in five pregnancies and varied between 21 and 23 days, counting the day of impregnation as the first day.

All 10 pregnancies resulted in the birth of healthy appearing fetuses. The birth weights of the fetuses are given in Table 5 both for the experimental and control pregnancies. There is no evidence from

the data in Table 5 that the fetuses resulting from the diabetic pregnancies had birth weights which were different from those born to normal rats. There was one litter in the experimental group containing 11 fetuses which averaged 6.4 grams per fetus. It probably is not significantly greater than the expected normal weight, since it is very close to the maximum weight of 6.5 grams which was found in one of the litters containing 10 fetuses born to a normal rat.

DOGS

Seven dogs were bred and injected with alloxan. Two dogs failed to develop glycosuria after repeated injections of 50 to 125 mgm. of alloxan per kilo of body weight. The remaining five developed diabetes. One died and a second dog remained sterile. The three remaining diabetic bitches were pregnant. The protocols of their pregnancies follow.

Dog 3. Fifty mgm. of alloxan per kilo of body weight was injected on the 19 day of pregnancy into this dog, which weighed 9.6 kg. During the remainder of the pregnancy there was an average of 20 grams of sugar excreted in the urine daily with a daily range from 6 to 70 grams. An average of 7 units of protamine zinc insulin was given each day. On the 52 day of pregnancy one small dead fetus was found in the cage.

Dog 5. Fifty mgm. of alloxan per kilo was injected on the 23 day of pregnancy into this dog, which weighed 11 kg. An average of 7 grams of sugar was excreted in the urine daily with a daily range from 0 to 40 grams. Four to seven units of protamine zinc insulin was given irregularly on 7 occasions. Delivery occurred on the 62 day of pregnancy. Three puppies were born alive and their average weight was 330 grams.

Dog 6. Fifty mgm. of alloxan per kg. was injected on the 21 day of pregnancy into this dog, which weighed 10 kg. The glycosuria amounted to 4 grams of sugar a day on the average with a range from 0 to 30 grams. No insulin was given. A litter of 7 puppies, all healthy in appearance, was born on the 61 day of pregnancy. Their average weight was 220 grams.

DISCUSSION

The experiments with the rabbits showed more similarity to some of the phenomena observed in human diabetic pregnancies than did those with the rats or dogs. The fact that about half of the diabetic pregnancies in the rabbits were associated either with late abortion and reabsorption of the fetuses or premature delivery or stillbirth is quite in keeping with the late accidents that occur in human diabetic pregnancies. Until recently the high stillbirth rate and neonatal mortality rate in human diabetic pregnancies have been attributed to a failure to regulate the maternal diabetes satisfactorily. This theory

has largely been superseded, because it has been shown that the incidence of stillbirths and neonatal deaths is very high for several years before clincial evidence of diabetes is found (Miller, 1945a) (Miller, Hurwitz and Kuder, 1944) (Allen, 1939). Miller has been able to show that a high fetal mortality occurs even though diabetes does not appear in the mother until after the child-bearing period is largely past (Miller, 1945b). Furthermore, there is some evidence that the high fetal and neonatal mortality rates in diabetic pregnancies are related to pathologic alterations in estrogenic and gonadotropic hormones in the mother during pregnancy (White and Hunt, 1943) (Smith, Smith and Hurwitz, 1944) (Smith, et al., 1946) (Rubin, Dorfman and Miller, 1946). It is true that the failure to regulate the diabetes of the mother will lead to a greater fetal mortality rate, witness the higher fetal mortality rates in the days before insulin and in pregnancies complicated by diabetic acidosis (Joslin, 1940). But the infant and fetal mortality rates are still about twenty per cent even among the offspring of mildly diabetic mothers who do not require insulin for satisfactory control (Miller, et al., 1944). Not all of the deaths among the fetuses in the experiments with rabbits can be attributed to a failure to keep the glycosuria within reasonable limits, since two of the pregnancies in the diabetic rabbits occurred at a time when glycosuria was minimal and had been so for several days preceding the termination of pregnancy.

The failure to demonstrate any peculiarities in the diabetic pregnancies in the rats is of interest chiefly because Long had an opposite experience with the same strain of rats, in which the diabetes was produced by removing as much of the pancreas as possible by surgical means when the rats were immature, weighing about 75 grams at the time of operation (Long). In 39 pregnancies in 15 diabetic rats Long observed that all of the pregnancies ended with reabsorption of the fetuses between the 11 and 19 day of pregnancy. Reabsorption was detected by noting the presence of gross blood clots in the vaginal discharge. All of the rats studied by Long had severe diabetes, excreting several grams of sugar a day in the urine, up to 13 grams a day in one rat. By comparison the rats in the present study would have to be considered as having mild diabetes and perhaps this difference offers an explanation for the divergent results.

The experiments with the dogs did not prove to be very satisfactory. In only one of the three diabetic dogs was there an abnormal pregnancy. The relatively long gestation period, the uncertainty as to whether or not the dogs were pregnant and the failure to induce glycosuria in two out of seven dogs made these animals less satisfactory than rabbits to deal with. One interesting fact did appear from the experiments on the dogs and that is the injection of alloxan per se did not seem to have any harmful effect on the fetuses. One dog which failed to develop significant glycosuria received increasingly

large injections of alloxan up to 125 mgm. per kg. between the 35 and 52 days of pregnancy on five separate occasions and delivered a healthy appearing litter of 8 puppies on the 63 day of pregnancy.

SUMMARY

Diabetic pregnancies in rabbits resulted in many abortions, premature deliveries, stillbirths and the delivery of immature fetuses. About half of the diabetic pregnancies were full-term. No evidence of an increased birth weight among the fetuses of diabetic rabbits was found.

All of the pregnancies of diabetic rats were full-term and resulted in the birth of healthy fetuses that had normal birth weights.

The experiments with diabetic dogs were too few to be conclusive. There was only one abnormal pregnancy in the three that were studied. The injection of alloxan during pregnancy appeared to have no ill effect on the fetuses.

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EFFECT OF THYROIDECTOMY ON THE CONVERSION OF CAROTENE TO VITAMIN A[1]

VICTOR A. DRILL AND ALDO P. TRUANT
From the Department of Pharmacology, Yale University School of Medicine
NEW HAVEN, CONNECTICUT

THERE is some indirect evidence in the literature indicating that the conversion of carotene to vitamin A may be impaired during experimental hypothyroidism (Drill, 1943). In 1926 Kunde noted the appearance of xerophthalmia in rabbits 8 to 12 months after thyroidectomy, even though the diet was adequate for normal (unoperated) animals. Other studies on carotene and vitamin A excretion in the milk of thyroidectomized animals also lead to the conclusion that the thyroid gland was necessary for the conversion of carotene to vitamin A (Fellenberger and Greuter, 1932; Fasold and Heidemann, 1933).

These papers suggest strongly that the conversion of carotene to vitamin A is not normal in the thyroidectomized animal. It remained, however, to test this hypothesis by using purified diets, and observing whether thyroidectomized animals receiving carotene but no vitamin A would develop signs of vitamin A deficiency. In the experiments reported herewith it was found that thyroidectomized rats receiving only carotene as a source of vitamin A developed signs of vitamin A deficiency.

METHODS

Female rats of the Sprague-Dawley strain weighing between 40 and 50 grams were fed a vitamin A-free diet consisting of: casein (vitamin-free), 18; salt mixture (U.S.P. No. 2), 4; starch, 64; Crisco, 5; yeast,[2] 8; and calcium lactate, 1%. Calcium lactate was added to the diet to prevent possible death following thyroidectomy because of the incidental removal of parathyroid tissue. Five U.S.P. units of vitamin D were added per gram of diet. Some of the animals on the vitamin A-free diet received either crystalline carotene or crystalline vitamin A subcutaneously in sesame oil every 2 days. The carotene contained 90 per cent beta and 10 per cent alpha carotene.

Forty-seven rats were divided into 6 groups, of which each group averaged between 44.6 and 45.8 grams in weight. Two days later they were all placed on the vitamin A-free diet and some of the animals were thyroidectomized. A number of the unoperated and thyroidectomized rats were continued on the vitamin A-free diet as controls; whereas others received

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injections of 10 gamma of carotene or 5 gamma of vitamin A every 2 days. (Table 1, Figure 1.).

RESULTS

Ophthalmological Changes. Unoperated rats. The unoperated animals receiving the vitamin A-free diet plus supplements of vitamin A or carotene are omitted from Table 2 to conserve space. The 6 animals receiving vitamin A supplements had normal eyes throughout the study. Of the six rats receiving supplements of carotene (Table 1), one showed some exudate on the 76th day of the experiment and a reddening of the lids and slight opacity between the 81st and 84th days, although she continued to gain weight. All of the unoperated animals remaining on the vitamin A-free diet without supplements developed eye lesions characteristic of vitamin A deficiency (Table 2).

Thyroidectomized rats. Thyroidectomized animals not receiving supplements of vitamin A or carotene developed the characteristic lesions of vitamin A deficiency. Vitamin A supplements administered to the thyroidectomized group prevented the appearance of such

TABLE 1. OCULAR CHANGES AND MORTALITY OF UNOPERATED AND THYROIDECTOMIZED RATS RECEIVING CAROTENE OR VITAMIN A

Rat group	No. rats	No. died	% death	Eyes patho-logical	% of eyes patho-logical
1. Control, no. vit.	6	6	100	6	100
2. Thyroidect., no. vit.	6	6	100	6	100
3. Control, +vit. A.	6	0	0	0	0
4. Thyroidect., +vit. A.	6	1	16	0	0
5. Control, +carotene	6	0	0	1	16
6. Thyroidect., +carotene	17	8[1]	47[1]	16	94

[1] Data include rats 39, 40, 42, 43, 46 and 47 only up to 58th day, as they received daily injections of thyroxin from this date. Excluding these 6 rats from the figures would give a mortality of 8 out of 11 animals during the 84 days of experiment, or 73% mortality.

ocular lesions (Tables 1 and 2). Thus, although carotene could prevent the appearance of ocular lesions in unoperated rats receiving a vitamin A-free diet, it failed to do so in the thyroidectomized group. This finding is in contrast to the protective effect of vitamin A supplements in both unoperated and thyroidectomized rats fed a diet devoid of vitamin A.

Weight changes. The control rats receiving vitamin A or carotene, respectively, showed comparable and constant gains in weight throughout the experiment whereas the group kept as controls and not receiving either substance showed but little gain in weight after the *12th* day (Fig. 1.). All of the thyroidectomized rats gained weight much more slowly. Among the operated groups the thyroidectomized rats receiving vitamin A had the greatest gain in weight; whereas the thyroidectomized animals lacking vitamin A or carotene showed the

lowest weight curve. The thyroidectomized group treated with carotene had a weight curve comparable to the thyroidectomized animals on the vitamin A-free diet and significantly different from that of the animals receiving vitamin A. That these two groups showed similar weight curves is to be expected, because the carotene treated animals developed the same ocular changes as vitamin A-deficient rats. Beginning at the 45th day, however, the carotene-treated animals tended to gain weight progressively and to differ from the vitamin A-deficient rats. One must judge from this finding that after the *45th*

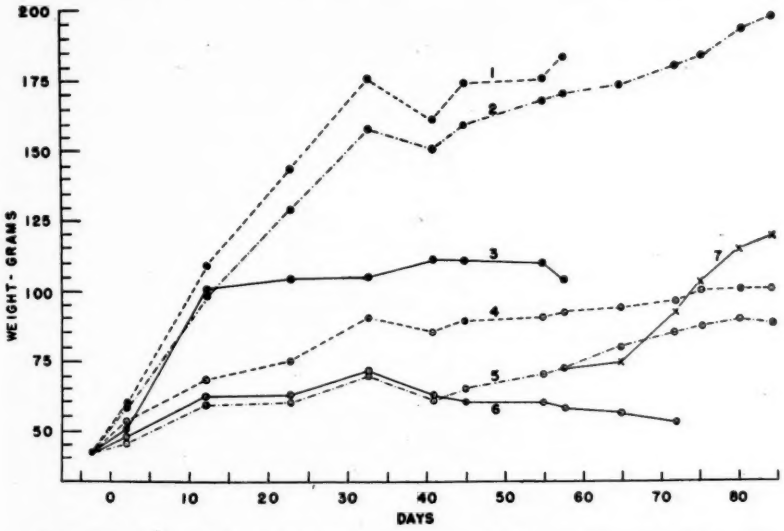

FIG. 1. Relation of thyroid gland to carotene metabolism. Groups 1, 2 and 3 unoperated animals. Groups 4, 5, 6 and 7 thyroidectomized at zero time. The following supplements were administered: Groups 1 and 4, vitamin A; Groups 2 and 5, carotene; Groups 3 and 6, neither carotene nor vitamin A; Group 7, carotene supplements, with thyroxin injections.

day the thyroidectomized animals treated with carotene were utilizing at least some of the carotene.

Mortality. The mortality of the various groups is summarized in Table 1. It should be noted that some of the thyroidectomized animals treated with carotene survived to the end of the study (Table 2). This is in keeping with the impression gained from a study of the weight curves, namely, that the rats surviving past the *45th* day must have utilized some of the carotene. Nevertheless, their eye signs did not improve during this period.

Thyroxin treatment. Because supplements of carotene failed to prevent signs of xerophthalmia in thyroidectomized rats, it was decided to ascertain whether injections of thyroxin would improve

TABLE 2. RELATION OF OCULAR CHANGES TO THYROID GLAND AND SUPPLEMENTS OF VITAMIN A OR CAROTENE

Days	33	38	41	45	52	55	58		65	72	76	81	84
Rat No.	Unoperated, vitamin A deficient												
13	+	++D											
14	0	+	0	0	0	++	++++D						
15	+D												
16	+++	+++	0	++++	++++	D							
17	++	+	++	+	D								
18	0	0	+++	0	++++	++++	++++D						
	Thyroidectomized, vitamin A deficient												
19	+	0	0	++++	+	++++	++++		++++	D			
20	+	0	+	++++	++++	++++	++++		D				
21	+	+	+	++	+++	++++	++++		++++	D			
22	0	0	0	++++	D								
23	+	+	+++	++++	++++	++++	++++		++++	++++	D		
24	+	+	++	D									
	Thyroidectomized, vitamin A deficient + vitamin A												
25	0	0	0	0	0	0	0		0	0	0	0	0
26	0	0	0	0	0	0	0		0	0	0	0	0
27	0	0	0	0	0	0	0		0	D			
28	0	0	0	0	0	0	0		0	0	0	0	0
29	0	0	0	0	0	0	0		0	0	0	0	0
30	0	0	0	0	0	0	0		0	0	0	0	0
	Thyroidectomized, vitamin A deficient + carotene												
31	+	+	0	+	+++	++++	++++		++++	++++	++++	++++	++++
32	+	+	+	+	+	++++	++++D						
33	+	+	+++	++++	++++	++++	++++		++++	D			
34	+	++	0	+	+	+	+	No thyroxin	+	++++	++++	++++	++++
35	+	+	0	D									
36	0	+++	D										
37	0	0	0	++++	D								
38	+	0	+	+	++	++++	++++		++++	++++	++++	++++	++++
39	0	0	0	++++	++	0	++++		++++	++++	++++	++++	++++
40	++++	++++	++++	++++	++++	++++	++++		++++	++++	++++	D	
41	0	0	0	++++	+	++++	D						
42	+	0	+	+	+	++++	++++	Thyroxin	++++	++++	0	0	0
43	+	+++	++	++++	++++	++++	++++	Injected	++++	++++	++++	++++	++++
44	+	+	++++	++++	++++	D							
45	+	+	+	D									
46	0	0	0	++++	+	++++	++++		++++	++++	++++	++++	0
47	0	0	0	++++	++++	++++	++++		++++	++++	++++	0	0

0 = normal eye; + = watery; ++ = swollen; +++ = bloody exudate; ++++ = opacity or purulent; D = death.

the ocular lesions. Starting on the *58th* day of the experiment daily injections of 5 gamma of thyroxin were administered to six of the animals. Of the rats that survived to the *84th* day, 3 of 3 untreated rats showed no change in ocular signs, whereas 3 of 5 animals treated with thyroxin showed a definite improvement in the ocular changes. Indeed the eyes returned completely to normal. Because of the small number of animals at this point of the study, the effect of thyroxin in enabling the thyroidectomized animals to metabolize carotene and to reverse the ocular changes is only suggestive and not conclusive.

DISCUSSION

Through the use of vitamin A-free diets it was demonstrated that supplements of carotene prevented the ocular changes characteristic of vitamin A deficiency in control rats, but failed to prevent xerophthalmia in thyroidectomized animals. The weight curve of the thyroidectomized rats receiving carotene was also similar to that of thyroidectomized control rats on a vitamin A-free diet. This finding also indicated that the carotene was not being utilized in the operated animals. The mortality was also increased in the carotene treated rats. Supplements of vitamin A prevented the ophthalmic lesions in both control and thyroidectomized rats, and such animals also showed a greater gain in weight. Although the thyroidectomized rats receiving carotene did not utilize sufficient carotene to prevent xerophthalmia from occurring, there still appeared to be a slight utilization of the carotene. This was inferred from the slight but progressive increase in weight after the *42nd* day and a more prolonged survival of some of the animals in this group.

This effect of thyroidectomy on the utilization of carotene is in agreement with previous suggestions and conclusions in this field. Kunde (1926) had noted the appearance of xerophthalmia in thyroidectomized rabbits. Later, it was reported that the milk of thyroidectomized goats was yellow, a finding which was attributed to the presence of a lipochrome, probably carotene (Fellenberger and Greuter, 1932). It was then demonstrated that thyroidectomy in goats increased the amount of carotene and decreased the vitamin A content of the milk (Fasold and Heidemann, 1933).

Two groups of authors have reported that carotene administered parenterally is not utilized as well as injected vitamin A. This feature was studied by the survival time after the administration of each substance was stopped and by the concentration of vitamin A and carotene in the liver (Lease, Lease, Steenbock and Baumann, 1942; Sexton, Mehl, and Deuel, 1946). Although this difference does exist it does not enter into the present experiment, as the dose of carotene used was sufficiently high so that the control rats utilized it effectively enough to gain weight practically as well as control animals receiving vitamin A.

SUMMARY

Supplements of vitamin A administered to rats receiving a diet devoid of vitamin A prevented the appearance of xerophthalmia in both control and thyroidectomized animals. However, supplements of carotene prevented the ocular changes only in the control rats and not in thyroidectomized rats. The mortality and changes in weight of the thyroidectomized animals receiving carotene also indicated a decreased utilization of carotene. It is apparent that the thyroid gland plays a major role in the conversion of carotene to vitamin A.

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REGULATION OF PITUITARY ADRENOCORTICOTROPHIC ACTIVITY DURING THE RESPONSE OF THE RAT TO ACUTE STRESS[1]

GEORGE SAYERS AND MARION A. SAYERS

From the Department of Pharmacology, University of Utah School of Medicine

SALT LAKE CITY, UTAH

INTRODUCTION

THE EXCITATORY effect of non-specific stress upon the secretory activity of the adrenal cortex is mediated through the anterior pituitary. The effect is manifested promptly, there being an appreciable reduction in the ascorbic acid concentration of the adrenal within an hour following the beginning of exposure of the organism to noxious stimuli. The present paper[2] is concerned with the mode of action of acute stress in modifying the rate of elaboration of adrenocorticotrophic hormone (A.C.T.H.) by the anterior pituitary.

Previous studies on the regulation of pituitary adrenocorticotrophic activity have been limited to periods of stress long enough to affect measurable change in adrenal size. Chronic administration of cortical extract (Ingle, Higgins and Kendall, 1938) or desoxycorticosterone acetate (Selye, 1940; Sarason, 1943) results in atrophy of the adrenal cortex. The locus of this suppressive action appears to be the anterior pituitary rather than the adrenal cortex, since the inhibitory effect of adrenal cortex extract can be counteracted by the simultaneous administration of A.C.T.H. (Ingle, Higgins and Kendall, 1938). Ingle (1938) was able to demonstrate an increase in adrenal size following a 12-hour period of muscular exercise in the rat. Hypertrophy did not occur in those animals treated with adrenal cortical extract during the period of exercise. The experiment may be interpreted to mean that administration of cortical extract abolished the normal stimulus to pituitary activity, namely, a decrease in concentration of cortical hormone(s) in the body fluids.

In the present study changes in pituitary adrenocorticotrophic activity occurring within a period of one hour or less, following subjection of an animal to stress, were measured by the alterations in the ascorbic acid content of the adrenal. This method for the determina-

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[2] Preliminary reports of this work have been published (Sayers and Sayers, 1945; Sayers and Sayers, 1946).

tion of pituitary adrenocorticotrophic activity is based upon the following facts. (a) Administration of purified A.C.T.H. or subjection to stress produced depletion of adrenal ascorbic acid within a period of less than one hour, and the amount of the depletion is proportional to the dose of A.C.T.H. or the degree of stress to which the animal is subjected (vide infra). (b) Ablation of the pituitary leaves adrenal ascorbic acid inert and non-responsive to stress (Sayers, Sayers, Liang and Long, 1945). (c) Purified A.C.T.H. free of other pituitary activities depletes the ascorbic acid of the adrenal of the hypophysectomized rat (Sayers and Sayers, 1945).

TECHNIQUE AND METHODS

Adult male rats of the Sprague-Dawley strain were used in all of the experimental procedures. They were maintained at an environmental temperature of $28 \pm 1°C.$ for at least a week before and at all times during the experimental period, with the exception of those animals subjected to heat or cold as a stress. In these latter experiments the animals were removed to the stressful temperature one hour prior to autopsy. The rats were fed a standard diet[3] ad libitum until 24 hours before autopsy at which time only water was made available. At the end of the period of stress the adrenals were removed under sodium pentobarbital anesthesia, carefully dissected free of fat, weighed to the nearest 0.1 mg. and analyzed for total ascorbic acid content by the method of Roe and Kuether (1943). It is not necessary to separate cortex from medulla since the alterations in the concentration of ascorbic acid in the whole adrenal reflect changes in the cortex (Sayers, Sayers, Liang and Long, 1946). Each rat subjected to heat was placed in a two quart jar submerged in a water bath maintained at 38°C. Fresh air was passed through the jar under a pressure of 5 cm. of water. Animals subjected to cold were placed either individually or in pairs in small wire baskets in a refrigerator maintained at the desired temperature. Histamine acid phosphate, a formalin killed suspension of whole typhoid organisms, and epinephrine were administered intraperitoneally in a volume of 0.9 per cent sodium chloride equal to 0.2 ml. per 100 gm. of body weight.

The crystalline steroids and adrenal cortical extract (Upjohn) were administered subcutaneously. The volume of injected material was in most instances equal to 0.4 ml. per 100 gm. of body weight and was divided equally among three sites of injection, the nape of the neck and both flanks. Each site of injection was rubbed vigorously for one minute. The one exception to this method of injection of cortical hormones was in the case of administration of 17-hydroxycorticosterone to animals treated with epinephrine. These animals were anesthetized with sodium pentobarbital during the period of hormone administration and the 17-hydroxycorticosterone was injected intravenously (by tail vein) in 10.5 ml. of 0.9 per cent sodium chloride solution. The injection was made over a period of 35 minutes at the rate of 0.1 ml. per 20 seconds, beginning 5 minutes before the injection of epinephrine.

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[3] 94% calf builder meal, 3% dried brewers' yeast and 3% wheat germ.

RESULTS

1. *General nature of the phenomenon*

Exposure to cold and heat, and injection of histamine, epinephrine and killed typhoid organisms brought about a reduction in the concentration of ascorbic acid in the adrenal of the rat (table 1). This reduction in the vitamin C content of the adrenals indicates that each

TABLE 1. INHIBITION OF PITUITARY ADRENOCORTICOTROPHIC ACTIVITY BY ADRENAL CORTEX HORMONE FOLLOWING SUBJECTION OF RATS TO A VARIETY OF STRESSES

Treatment	No. of rats	Pretreatment with adrenal cortex extract injected subcutaneously ml. per 100 gm. body wt.	Adrenal ascorbic acid mg. per 100 gm. fresh tissue. Ave. and standard error
Controls	27 6	None 1.1	419 ± 6.6 423 ± 8.4
Cold[1] (exposed to 3–5°C for one hour before sacrifice)	9 9	None 1.4	314 ± 18.0 415 ± 10.1
Heat (exposed to 38°C for one hour before sacrifice)	11 6	None 1.0	277 ± 16.7 405 ± 10.9
Histamine (0.5 mg. per 100 gm. body wt. intraperitoneally one hour before sacrifice)	11 8	None 0.1 mg.[2]	265 ± 9.7 422 ± 13.6
Epinephrine (4.0 micrograms per 100 gm. body wt. intraperitoneally one half hour before sacrifice)	3 3	None 0.1 mg.[3]	308 419
Typhoid organisms (0.2 ml. of a formalin killed suspension per 100 gm. body wt. intraperitoneally one hour before sacrifice)	7 8	None 0.5	305 ± 17.3 441 ± 13.8
Adrenocorticotrophic hormone (10 micrograms intraperitoneally one hour before sacrifice)	4 6	None 1.5	314 ± 12.1 310 ± 7.3

[1] Rats placed in pairs in small wire basket in refrigerator.
[2] 17-hydroxycorticosterone subcutaneously.
[3] 17-hydroxycorticosterone intravenously.

one of these stresses acting for a period of one hour stimulated the anterior pituitary to elaborate A.C.T.H. On the other hand, if the animals were administered cortical hormone prior to the application of the stress, no reduction in the concentration of adrenal ascorbic acid took place. Hence, administration of cortical hormone acted to inhibit the increase in adrenal cortical activity which normally follows application of noxious stimuli.

2. *Locus of action of adrenal cortical hormone*

As can be seen from table 1, the amount of hog A.C.T.H. which when injected intraperitoneally produced the same reduction in adrenal ascorbic acid as exposure to 3-5°C for one hour, was found to be equal to ten micrograms per 100 gm. of body weight. Hence, the

amount of hormone elaborated from the anterior pituitary during exposure to this temperature for one hour has the activity equivalent to ten micrograms of hog A.C.T.H. Whereas pretreatment with adrenal cortical hormone prevented the reduction in adrenal ascorbic acid which otherwise follows exposure to cold, it had no effect whatsoever upon the adrenal-stimulating effect of the exogenous source of A.C.T.H. (table 1). Hence, the locus of the inhibitory action of adrenal cortical hormone is not the adrenal cortex itself. By elimina-

TABLE 2. QUANTITATIVE ASPECTS OF INHIBITION OF PITUITARY ADRENOCORTICOTROPHIC ACTIVITY

Temperature to which rats were exposed for one hour	No. of rats	Pretreatment with adrenal cortex extract injected subcutaneously ml. per 100 gm. of body wt.	Adrenal ascorbic acid mg. per 100 gm. fresh tissue. Ave. and standard error
Unexposed controls	27	None	419 ± 6.6
7±1°C	7	None	365 ±18.7
4±1°C	21	None	273 ±10.9
	8	0.01	287 ±25.5
	12	0.1	354 ±21.6
	8	0.2	406 ±19.3
	9	0.3	404 ±15.6
−2±1°C	6	None	242 ±12.9
−8±1°C	5	None	213 ± 7.84
	4	1.0	254 ± 7.53

tion it would appear that adrenal cortical hormone acts to suppress elaboration of A.C.T.H. from the anterior pituitary.

3. *Quantitative relationships*

Reducing the temperature of exposure successively from 7±1°C to 4±1°C, to -2±1°C and to -8±1°C brought about correspondingly greater reductions in adrenal ascorbic acid (table 2). Likewise, when the dose of injected histamine was increased successively from 0.25 mg. to 0.5 mg., to 1.0 mg. and to 10.0 mg. per 100 gm. body weight, correspondingly greater reductions in the ascorbic acid content of the adrenals took place (table 3). Furthermore, there is a direct relationship between the dose of A.C.T.H. administered and the reduction in adrenal ascorbic acid (table 4). Hence, the amount of adrenocorticotrophic hormone elaborated from the pituitary is proportional to the intensity of stress to which the animal is exposed.

The degree of inhibition of pituitary adrenocorticotrophic activity is proportional to the amount of administered cortical hormone. As can be seen from table 2, 0.3 and 0.2 ml. of cortical extract per 100 gm. of body weight prevented the reduction in adrenal ascorbic acid which

TABLE 3. COMPARATIVE PITUITARY INHIBITORY POTENCY OF FIVE CRYSTALLINE STEROIDS

Stress		No. of rats	Steroid	Dose of steroid injected subcutaneously micrograms per 100 gm. body wt.	Adrenal ascorbic acid mg. per 100 gm. fresh tissue ave. and standard error	Estimated pituitary inhibitory potency
Controls—no stress		27	None	—	419± 6.6	
4±1°C for one hour		21	None	—	273±10.9	
		8	17-hydroxycorticosterone	50	426±10.9	4
		11		10	350±23.6	
		7	corticosterone	200	444±18.1	1
		8		50	360±21.6	
		12		50[1]	366±20.2	
		8	desoxycorticosterone	200[1]	401±22.6	½
		8		50[1]	299±12.9	
		5	progesterone	10,000[1]	437±13.6	1/50
		4		1,000[1]	312±46.4	
Histamine mg. per 100 gm. body wt. intraperitoneally one hour before sacrifice		13	None	—	350±16.8	
	0.25	4	17-hydroxycorticosterone	10	394±28.8	
		9		20	417±10.9	
		4	17-hydroxy-11-dehydro-corticosterone	20	390±14.1	
	0.5	11	None	—	265± 9.7	
		10	17-hydroxycorticosterone	20	306±11.8	
		5		50	352±31.7	4
		8		100	422±13.6	
		7	17-hydroxy-11-dehydro-corticosterone	50	356±22.0	
		7		100	397±15.2	4
		2		200	429	
	1.0	6	None	—	229± 9.4	
		3	17-hydroxycorticosterone	500–1000	337±16.3	
	10.0	4	None	—	175±10.1	

[1] Olive oil was the vehicle. In all other cases the vehicle was 10 per cent ethanol in 0.9 per cent NaCl.

TABLE 4. EFFECT OF ADMINISTRATION OF A.C.T.H. (HOG) UPON ADRENAL ASCORBIC ACID

Dose of A.C.T.H. micrograms per 100 gm. body wt.	No. rats	Adrenal ascorbic acid[1] mg. per 100 gm. fresh tissue. Average and standard error
0	27	419±6.6
2.5	12	297±4.2
10.0	6	250±9.1

[1] Adrenals removed one hour after hormone administration.

followed exposure of rats to 4±1°C. On the other hand, 0.01 ml. had very slight if any, and 0.1 ml. had only partial, blocking effect.

The data in table 3 illustrate that *the greater the degree of stress to which the animal is subjected the greater is the amount of cortical hormone required to inhibit pituitary adrenocorticotrophic activity.* Whereas 20 micrograms of 17-hydroxycorticosterone per 100 grams of body weight completely inhibited the increase in adrenal cortical activity which otherwise follows injection of 0.25 mg. of histamine, this dose of steroid produced only partial inhibition in rats treated

with 0.5 mg. of histamine. However, the greater pituitary adrenocorticotrophic activity produced by this larger dose of histamine could be blocked by administration of 100 micrograms of 17-hydroxycorticosterone. In animals treated with 1.0 mg. of histamine, a dose of 1.0 mg. of 17-hydroxycorticosterone resulted in only partial suppression of pituitary activity.

4. *Relative pituitary inhibitory potency of crystalline steroids*

An estimate of the pituitary inhibitory potency of five crystalline steroids has been made. The approximate potency rating has been assigned on the basis of the comparative ability of these substances to block the pituitary adrenocorticotrophic activity which accompanies exposure to a uniform degree of cold or which follows injection of a standard dose of histamine (table 3). Seventeen-hydroxycorticosterone was the most potent steroid in blocking pituitary adrenocorticotrophic activity which normally follows exposure to cold (one hour at $4 \pm 1°C$). This compound has been assigned a potency rating of 4. Following in order of potency were corticosterone, desoxycorticosterone, and progesterone which have been assigned approximate potency ratings of 1, ½, and 1/50, respectively.[4] Seventeen-hydroxycorticosterone and 17-hydroxy-11-dehydrocorticosterone appear to be equally potent in blocking the pituitary stimulating effect of histamine.

DISCUSSION

The alteration in the concentration of ascorbic acid of the adrenal offers certain advantages as a measure of pituitary adrenocorticotrophic activity. Rapidly fluctuating rates of elaboration of A.C.T.H. can be detected. Changes in pituitary adrenocorticotrophic activity occurring within a period of one-half hour following injection of epinephrine have been observed. Furthermore, the quantitative nature of the method has made it possible to demonstrate that the rate of elaboration of A.C.T.H. from the pituitary is proportional to the intensity of stress to which the animal is subjected.

The administration of cortical hormone suppresses the enhanced adrenal cortical activity which normally accompanies environmental change (heat, cold), drug administration (histamine, epinephrine) and bacterial intoxication (injection of a suspension of killed typhoid organisms). In other words, if the animal is furnished with an adequate amount of adrenal cortical hormone during exposure to any one of a variety of stresses, the normal stimulus to adrenal cortical activity is abolished. It would appear reasonable to suppose that the great variety of non-specific stresses increases pituitary adrenocorti-

[4] The experiments were of short duration. A period of one hour elasped between injection of the steroid and subjection of the animal to stress. Hence the estimated potencies of these steroids are undoubtedly influenced by their relative solubilities in the vehicle as compared to the tissue fluids surrounding the subcutaneous site of injection.

cotrophic activity by a common mechanism, namely, by increasing the requirement of the peripheral tissue cells for cortical hormone(s). The anterior pituitary responds to the decrease in concentration of cortical hormone in the body fluids by increasing the rate of elaboration of A.C.T.H.[5]

Elaboration of A.C.T.H. in response to stress could be completely or partially blocked depending upon the dose of cortical hormone administered. Furthermore, with increasing intensity of stress, the amount of cortical hormone required to suppress pituitary adrenocorticotrophic activity became correspondingly greater. These quantitative data may be adequately explained if it is assumed that the rate of elaboration of A.C.T.H. from the pituitary can fluctuate by gradual change and according to the requirements of the tissue cells for cortical steroids. Just as the pituitary-thyroid system maintains the tissues in a state of well-being in regard to thyroid hormone (euthyroidism) (Salter, 1944), so the pituitary-adrenal system maintains the tissues in a state of well-being with regard to adrenal cortical hormone(s) ("eucorticism").

The interrelationships between the pituitary, the adrenal cortex and the tissue cells are diagrammatically presented in Figure 1.

Despite limitations in regard to solubility, desoxycorticosterone has one-eighth and one-half the pituitary inhibitory potency of 17-hydroxycorticosterone and corticosterone, respectively. Desoxycorticosterone produces sodium retention, and 17-hydroxycorticosterone produces sodiumphoresis (Thorn, Engel and Lewis, 1941). Quantitative studies (Olson, Thayer and Kopp, 1944) indicate that desoxycorticosterone acetate has no glycogenic activity in amounts 100 times as great as effective glycogenic doses of corticosterone. Desoxycorticosterone is further distinguished from the C_{11} oxygenated steroids in having no lymphocytolytic action (Dougherty and White, 1944). The fact that pituitary inhibitory potency resides in compounds which are qualitatively distinct from each other in their metabolic activity suggests that the steroids act directly on the anterior pituitary rather than indirectly through some product of their metabolic activity or through their deficiency.

SUMMARY

The increase in the rate of elaboration of A.C.T.H. by the pituitary during exposure of the rat to stress for a period of one hour or less is proportional to the decrease in the concentration of adrenal ascorbic acid which accompanies such exposure.

[5] It is recognized that it may not be the pituitary itself which responds to the concentration of adrenal cortical hormones in the body fluids but rather some intermediary structure, as for example, the hypothalamus, which might possibly regulate anterior pituitary adrenocorticotrophic activity via nervous pathways. Uotila (1939), however, has demonstrated in chronic experiments that the adrenal cortical hypertrophy which occurs in rats exposed to cold is not influenced by transection of the infundibulum.

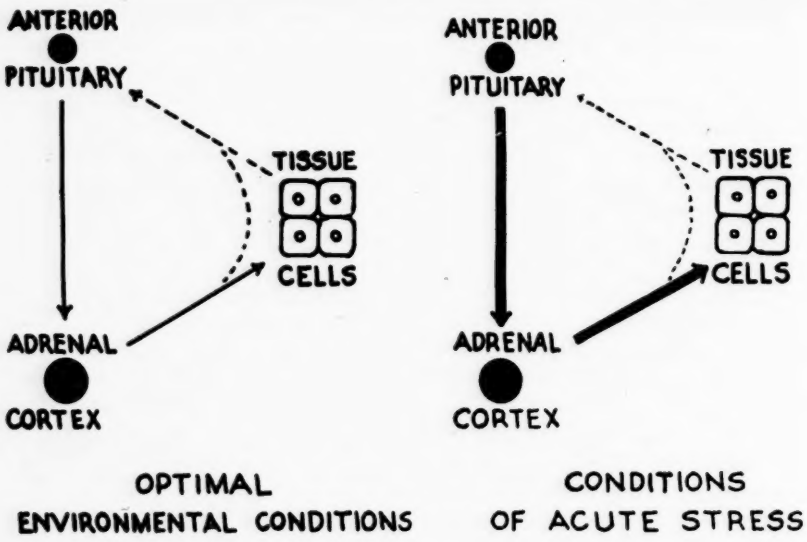

FIG. 1. The pituitary-adrenal system under optimal environmental conditions and under conditions of stress. The width of the vertical lines is proportional to the concentration of A.C.T.H. in the body fluids. The width of the other lines is proportional to the concentration of adrenal cortex hormone(s) in the body fuids. The two dashed lines (one off to the side of the tissue cells, and the other going directly from the tissue cells to the pituitary) indicate that the cortical hormone(s) may have a direct inhibitory effect on the pituitary or that they may have an indirect effect through some product of their metabolic action on the tissue cells.

Under optimal conditions of the environment, the pituitary-adrenal system is in a relatively quiescent state. The peripheral tissue cells under these conditions require relatively small amounts of cortical hormone(s), and the concentration of the latter in the circulating blood is maintained at a level which holds the anterior pituitary in check. This means that under optimal conditions of the environment minimum amounts of A.C.T.H. are elaborated by the anterior pituitary and minimum amounts of cortical hormone(s) are secreted by the adrenal cortex.

This is to be contrasted with the activity of the pituitary-adrenal system which occurs under conditions of stress. The requirement for cortical hormone(s) by the tissue cells is great, and hence the quantity in the circulating blood is reduced. The check on the anterior pituitary is now removed and A.C.T.H. is elaborated in relatively large quantities. Increased secretory activity of the adrenal cortex occurs, meeting the needs of the peripheral tissue cells for the cortical steroids. This increased activity of the pituitary-adrenal system continues until the stress is removed or until adaptation occurs, at which time the requirement of the peripheral tissue cells for cortical hormone(s) is diminished.

Adrenal cortical hormone, administered immediately before exposure of the rat to any one of a number of stresses (cold, heat, injection of histamine, epinephrine or killed typhoid organisms), suppresses the enchanced adrenal cortical activity which otherwise follows such an exposure.

Seventeen-hydroxycorticosterone, 17-hydroxy-11-dehydrocorticosterone, corticosterone, desoxycorticosterone and progesterone have

been assigned estimated pituitary inhibitory potency ratings of 4, 4, 1, ½, and 1/50, respectively.

The results have been interpreted to mean that the anterior pituitary elaborates A.C.T.H. at a rate inversely proportional to the concentration of cortical hormone(s) in the body fluids and according to the requirements of the peripheral tissue cells for cortical steroids.

The data suggest, but do not offer conclusive proof, that the cortical steroids act directly on the anterior pituitary rather than indirectly through products of their metabolic activity or their deficiency.

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THE ROLE OF THE ADRENAL CORTEX IN MAMMARY DEVELOPMENT AND ITS RELATION TO THE MAMMOGENIC ACTION OF THE ANTERIOR PITUITARY

A. T. COWIE AND S. J. FOLLEY

From the National Institute for Research in Dairying, University of Reading

READING, ENGLAND

THE POSSIBLE involvement of the adrenal cortex in mammary development was first indicated by the experiments of Van Heuverswyn, Folley and Gardner (1939) who showed that desoxycorticosterone promoted mammary duct growth in immature male mice. Later, though Chamorro (1945) working under rather different conditions observed no more than slight and irregular mammary duct stimulation in mice in response to desoxycorticosterone, it has been shown that desoxycorticosterone will evoke growth of the mammary alveolar tissue in the monkey (Speert, 1940), mouse (Mixner and Turner, 1942) and guinea pig (Nelson, Gaunt and Schweizer, 1943). Other crystalline adrenal cortex steroids have not yet been examined from this point of view, but in keeping with the foregoing results it may be noted that Edwards, Shimkin and Shaver (1938) observed gynecomastia in a man treated with cortical extract, while Nelson (1941) has observed mammary development in hypophysectomized-gonadectomized rats treated with adrenotrophin.

On the other hand, experiments on the effects of adrenalectomy on mammary development have given results which in certain respects are rather conflicting among themselves and moreover difficult to reconcile with the above findings since they indicate that the adrenal cortex may inhibit rather than promote growth of the mammae. Butcher (1939) claimed that the mammary glands of underfed adrenalectomized rats were larger and more arborescent than those of underfed controls; Reeder and Leonard (1944), however, could detect no increase in gland area after adrenalectomy in the rat but observed an increase in the number of lateral duct buds and branches which was most marked in those rats in which body growth was least inhibited.

The clear indication, however, that one at least of the specific adrenal cortex steroids will evoke mammary growth, taken in conjunction with the fact that the cortex is believed to be capable of secreting male and female sex hormones, the mammogenic properties

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of which are well known, made it advisable further to investigate the effects of adrenalectomy on the mammary gland. Extension of the rather meagre existing knowledge of the relation between the adrenal cortex and mammary development is particularly desirable in connection with a critical appraisal of current views according to which the anterior pituitary secretes mammogenic hormones which are the direct agents of mammary growth (Lewis and Turner, 1939; Mixner and Turner, 1943). Since it is likely that adrenotrophin is present in anterior-pituitary extracts which exhibit mammogenic properties it is

TABLE 1. DETAILS OF EXPERIMENTAL GROUPS

Number of rats ♂	Number of rats ♀	Code-lettering	Details of treatments
		Experiment 1. Gonadectomy and adrenalectomy on day 21.	
6	6	a. Adrenals intact:	no treatment.
5	6	b. Adrenals intact:	6 daily injections of 0.4 ml. A.P.[1]
6	6	c. Adrenalectomy:	no treatment.
4	5	d. Adrenalectomy:	6 daily injections of 0.4 ml. A.P.
		Experiment 2. Gonadectomy on day 23, adrenalectomy on day 61.	
7	6	a. Adrenals intact:	no treatment.
7	6	b. Adrenals intact:	6 daily injections of 0.4 ml. A.P.
8	6	c. Adrenalectomy:	no treatment.
8	6	d. Adrenalectomy:	6 daily injections of 0.4 ml. A.P.
		Experiment 3. Gonadectomy on day 27, adrenalectomy on days 74–85.	
7	5	a. Adrenals intact:	no treatment.
7	5	b. Adrenals intact:	10 daily injections of 0.4 ml. A.P.
6	5	c. Adrenalectomy:	no treatment.
6	4	d. Adrenalectomy:	10 daily injections of 0.4 ml. A.P.

[1] A.P. Ox anterior-pituitary extract (1 ml. = 0.25 gm. fresh tissue).
All injections were subcutaneous and began on the day following adrenalectomy, or on an equivalent day in the case of control rats.
The rats were autopsied 24 hours after the last injection, or if untreated, at corresponding times after adrenalectomy.

clearly necessary to ascertain the contribution of the adrenal cortex to the total mammogenic effect. A preliminary account of part of the work described in this paper has already been published (Cowie and Folley, 1944).

METHODS

Immature hooded Norway rats of both sexes were used, in all cases gonadectomized either at weaning (21 days) or a few days thereafter. The gonads were removed through dorsal incisions so as not to damage the mammary tissue. In one of the three experiments the adrenals were removed at the same time and through the same incisions; in the others adrenalectomy was postponed until some weeks after weaning. Adrenalectomized rats were given saline to drink instead of water.

Some groups were given, beginning on the day following adrenalectomy

or on an equivalent day in the case of control rats, daily subcutaneous injections of a saline extract of ox anterior pituitary (Young, 1941), 1.0 cc. of which was equivalent to 250 mg. fresh tissue. Details of the various treatments are given in Table 1.

The rats were killed with coal gas at the end of the experimental period and skinned. Each skin was pinned out on a cork sheet and fixed in Bouin's fluid, well washed in running water and the layer of subcutaneous fascia, containing the glands, dissected from the pelt by use of the blunt end of a scalpel handle. Where necessary, excess fat was removed by soaking in acetone and the glandular tissue stained with Mayer's haemalum followed by differentiation in acidified 70% alcohol. The individual glands were cut out of the sheet of connective tissue, dissected from as much muscle and connective tissue as possible under the low-power binocular, taken up through the alcohols into xylol and mounted on slides in Canada balsam. All of the glands from one rat, normally 12 in the female and 10 in the male, could be mounted on one or two slides depending on their size.

The area of each gland was estimated by tracing a line round the periphery on to Cellophane and the trace placed over millimeter squared paper. It was thus possible to estimate the total mammary gland area for each rat.

In order to make comparisons, which were as nearly objective as possible, of the effects of the various treatments on the structure of the mammary gland, a semi-quantitative method of scoring the kind and degree of glandular development was devised. The system of scoring was as follows:

Arborescence of duct system

D—Restricted duct system showing little branching; a few tertiary ducts present.

2D—Duct system of moderate extent showing intermediate degree of arborescence; many tertiary branches and also some of higher order.

3D—Widespread duct system, showing high degree of arborescence.

Club-shaped end buds, usually deeply staining

E—A few present

2E—Club-shaped end buds regularly disposed round the periphery.

Side buds

B—Very few present.

2B—Numerous side buds fairly evenly distributed.

3B—Ducts covered with side buds.

Alveolar development

A—Occasional alveoli present.

2A—Moderate alveolar development, evenly distributed.

3A—Ducts covered with alveoli.

Each mammary spread was examined under the binocular and assigned a score. For each morphological feature studied, the scores for each rat were totalled and the mean score per rat determined for each group. In practice, the slides from all of the rats of one sex in a given experiment were randomized and scored by both authors independently.

In the preliminary analysis of one of the experiments (Cowie & Folley 1944) missing glands were not assigned a mean score. It was later noted,

however, that glands were more frequently missing from adrenalectomized rats than from non-adrenalectomized ones owing to the greater difficulty in skinning these animals and therefore such losses were not randomly distributed throughout the groups. To minimise this error, missing or badly damaged glands have now been assigned the mean area value and score for that particular gland in the group in question. Since the average degree of mammary development varied considerably in the three experiments because of the different ages of the rats concerned, the scoring system had to some extent to be specially adapted to each experiment and thus allowed only of comparison between groups within any one experiment and not between groups in different experiments. Since side buds are potential ducts one would expect a high degree of correlation between the scores for duct development and the presence of side buds and it was therefore decided to combine the scores for these features.

The mammary gland areas and the scores for the above-mentioned morphological features were subjected to an analysis of variance[1] to determine the effect of (a) adrenalectomy and (b) treatment with anterior-pituitary extract on the mammary gland of the gonadectomized rat and (c) whether or not any effect of anterior-pituitary extract is modified by adrenalectomy. Since, as far as we are aware, this is the first attempt to apply statistical methods to the evaluation of changes in mammary gland development, it was considered worth while carrying out subsidiary statistical tests on the relative consistency of the two observers and the degree of correlation between their scores.

RESULTS

The mean mammary gland areas per rat for the various groups are given in Table 2 and the mean scores for mammary development in Table 3. The essential data relating to the analyses of variance of the areas and scores are also given in these tables.

EFFECT OF ADRENALECTOMY ON THE MAMMARY GLAND

Some slight evidence of mammary gland regression after adrenalectomy was obtained in Experiments 1 and 2 but not in Experiment 3 (see Tables 2 and 3). Only in the experiment involving the youngest rats, and in the males only, was the regression of sufficient degree to be reflected in a significant decrease in glandular area ($P=0.02$).

The possibility must be considered that since adrenalectomy is usually accompanied by a reduction of food intake and, in growing rats, of growth rate, the observed effects on the mammary gland may have been primarily due to this factor and not directly to the loss of adrenal cortex hormones. Daily body-weight data which are available for Experiments 1 and 2 indicate, however, that this is not the case. Whereas in Experiment 1, in which definite mammary regression occurred after adrenalectomy, the operation had little or no effect on the growth rate; in Experiment 2, in which the only observed mam-

[1] The statistical analysis of the results was carried out by The Scientific Computing Service Ltd., London, England.

TABLE 2. MEAN MAMMARY GLAND AREA (mm.²)

Treatments	Males				Females			
	a	b	c	d	a	b	c	d
Experiment								
1	235.5	231.6	176.6	219.7	284.8	321.7	292.2	375.2
2	450.0	572.9	443.9	467.4	740.7	817.7	857.0	713.0
3	632.1	571.4	535.4	459.0	919.4	1376.8	880.0	889.5

Statistical analysis

	Males				Females			
	I-A	b	c	d	I-A	b	c	d
Exp. 1								
M.D.	23.5	− 3.9	−58.9	− 15.8	23.05	36.9	7.4	90.4
S.E.	39.44	25.42	22.65	21.92	40.04	20.92	21.27	18.36
t	1.25	− 0.15	− 2.60	− 0.72	1.35	1.76	0.35	4.92
P	0.23	0.88	0.02	0.50	0.20	.10	0.74	0.00
Exp. 2								
M.D.	−49.7	122.9	− 6.1	17.4	−110.5	77.0	116.3	−27.7
S.E.	80.28	40.93	53.36	35.42	184.73	97.88	104.41	62.00
t	− 1.62	3.00	− 0.11	0.49	− 1.46	0.79	1.11	− 0.45
P	0.12	0.02	0.92	0.64	0.17	0.44	0.30	0.66
Exp. 3								
M.D.	− 7.85	−60.7	−96.7	−173.1	−223.95	457.4	−39.4	−29.9
S.E.	136.15	60.16	74.49	87.44	261.11	213.86	95.35	116.80
t	− 0.14	− 1.01	− 1.30	− 1.98	− 1.85	2.14	− 0.42	− 0.26
P	0.89	0.34	0.22	0.08	0.08	0.06	0.68	0.80

I-A—Inter-action between anterior-pituitary extract and adrenalectomy.
M.D.—Difference of the average area of experimental group b, c, and d, each taken against the control group a, except in the case of the Inter-action where

$$\text{M.D.} = \frac{(a+d)-(b+c)}{2}.$$

S.E.—Standard error.

mary change was a reduction in end-bud score in females reported by one observer only (P=0.04), body growth entirely ceased after the operation.

Though statistically significant changes have not invariably been observed following adrenalectomy in our experiments, when they have occurred they have always been in the direction of regression and our results are thus directly opposed in this respect to those of Butcher (1939) and Reeder and Leonard (1944).

MAMMOGENIC EFFECTS OF ANTERIOR-PITUITARY EXTRACT IN GONADECTOMIZED RATS WITH INTACT ADRENALS

Tables 2 and 3 show that some evidence of the presence of mammogenic activity in the anterior-pituitary extracts used was obtained in all three experiments. In Experiment 1, though there were no statistically significant changes in mammary gland area or duct scores, the presence of deeply-staining club-shaped end buds (indicative of

TABLE 3. MEAN MAMMARY GLAND SCORES (AVERAGE OF OBSERVERS A AND B)

A. Ducts and Side-Buds

Treatments	Males				Females			
	a	b	c	d	a	b	c	d
Experiment								
1	32.1	36.2	25.3	29.6	45.0	45.7	39.6	41.4
2	37.6	42.8	37.2	41.4	51.4	57.5	52.1	51.5
3	38.6	37.6	37.5	41.2	55.0	60.3	54.2	53.6

Statistical analysis

Observer A	Males				Females			
	I-A	b	c	d	I-A	b	c	d
Exp. 1								
M.D.	1.00	4.0	−7.2	−1.2	1.15	0.5	−5.2	−2.4
S.E.	3.82	1.98	2.29	1.84	2.91	1.59	1.68	1.39
t	0.59	2.02	−3.14	−0.65	0.95	0.31	−3.10	−1.73
P	0.58	0.08	0.02	0.54	0.35	0.76	0.02	0.12
Exp. 2								
M.D.	−1.15	6.8	0.1	4.6	−3.00	7.0	0.4	1.4
S.E.	5.16	2.33	2.95	2.91	6.29	3.29	1.75	4.03
t	−0.61	2.92	0.03	1.58	−1.16	2.13	0.23	0.35
P	0.55	0.02	0.98	0.14	0.27	0.06	0.82	0.74
Exp. 3								
M.D.	2.35	0.3	−2.6	2.4	−3.90	6.6	−1.4	−2.6
S.E.	5.51	2.43	2.91	3.01	4.63	2.38	2.42	3.38
t	1.08	0.12	−0.89	0.80	−1.82	2.77	−0.58	−0.77
P	0.30	0.90	0.40	0.44	0.09	0.02	0.58	0.46
Observer B								
Exp. 1								
M.D.	−0.75	4.2	−6.4	−3.7	−0.15	1.0	−5.4	−4.7
S.E.	3.55	2.04	2.25	1.58	3.78	1.95	1.90	2.22
t	−0.48	2.06	−2.84	−2.34	−0.10	0.51	−2.84	−2.12
P	0.63	0.08	0.02	0.04	0.92	0.62	0.02	0.06
Exp. 2								
M.D.	0.10	3.7	−1.0	2.9	−3.70	5.2	1.0	−1.2
S.E.	5.42	1.97	3.54	1.91	4.62	1.22	1.61	3.33
t	0.05	1.88	−0.28	1.52	−1.95	4.26	0.62	−0.36
P	0.96	0.08	0.78	0.16	0.07	0.00	0.56	0.72
Exp. 3								
M.D.	2.35	−2.3	0.3	2.7	−1.95	4.0	−0.2	−0.1
S.E.	5.10	2.47	2.38	2.73	3.70	2.05	1.98	2.59
t	1.17	−0.93	0.13	0.99	−1.14	1.95	−0.10	−0.04
P	0.26	0.38	0.90	0.34	0.28	0.08	0.92	0.96

B. Alveoli

Treatments	Males				Females			
	a	b	c	d	a	b	c	d
Experiment								
1	no alveoli present				no alveoli present			
2	2.2	4.5	1.6	3.1	5.7	3.8	5.2	6.4
3	6.0	12.6	2.4	6.1	3.3	8.2	3.6	8.0

TABLE 3—(*continued*)

Statistical analysis

Observer A	Males				Females			
	I-A	b	c	d	I-A	b	c	d
Exp. 2								
M.D.	−1.05	3.0	−0.4	0.5	1.65	−2.5	−1.0	−0.2
S.E.	3.03	1.57	1.51	1.50	3.60	2.28	1.68	1.64
t	−0.95	1.91	−0.26	0.33	1.12	−1.10	−0.60	−0.12
P	0.35	0.08	0.80	0.74	0.28	0.30	0.56	0.90
Exp. 3								
M.D.	−0.85	6.0	−4.5	−0.2	0.70	6.2	0.4	8.0
S.E.	6.12	3.48	3.18	3.67	4.24	2.72	2.47	3.08
t	−0.35	1.72	−1.42	−0.05	0.36	2.28	0.16	2.60
P	0.73	0.12	0.18	0.96	0.72	0.06	0.88	0.04
Observer B								
Exp. 2								
M.D.	0.15	1.7	−0.6	1.4	1.35	−1.2	0.1	1.6
S.E.	2.15	1.24	1.20	1.17	2.60	1.05	1.48	1.3b
t	0.19	1.37	−0.50	1.20	1.27	−1.14	0.07	1.19
P	0.85	0.20	0.62	0.26	0.22	0.28	0.94	0.26
Exp. 3								
M.D.	−1.9	7.0	−2.9	0.3	−1.2	3.6	0.2	1.4
S.E.	6.64	3.20	1.58	2.69	2.20	1.69	0.47	0.95
t	−0.73	2.19	−1.84	0.11	−1.18	2.13	0.43	1.47
P	0.48	0.04	0.10	0.92	0.27	0.06	0.68	0.18

C. End-Buds

Treatments	Males				Females			
	a	b	c	d	a	b	c	d
Experiment								
1	1.5	9.8	1.0	3.6	4.1	12.1	8.2	15.6
2	0.2	1.0	0.0	0.4	0.6	2.4	0.1	1.5
3	0.0	2.3	0.0	1.0	0.0	1.0	0.0	3.5

Statistical analysis

Observer A	Males				Females			
	I-A	b	c	d	I-A	b	c	d
Exp. 1								
M.D.	−3.05	7.8	0.0	1.7	−0.45	8.5	3.6	11.2
S.E.	3.47	2.71	0.67	0.71	4.15	2.13	2.49	2.44
t	−1.99	2.88	0.00	2.39	−0.26	3.99	1.45	4.59
P	0.07	0.02	1.00	0.04	0.80	0.00	0.18	0.00
Exp. 2								
M.D.	0.25	0.0	0.0	0.5	−0.40	1.5	−0.5	−0.2
S.E.	0.46	0.00	0.00	0.35	2.18	1.65	0.22	0.70
t	1.49			1.43	−0.45	0.91	−2.27	−0.29
P	0.15			0.18	0.65	0.38	0.04	0.78
Exp. 3								
M.D.	−0.55	2.0	0.0	0.9	0.85	0.8	0.0	2.5
S.E.	1.54	0.93	0.00	0.71	1.64	0.58	0.00	1.32
t	−0.94	2.15		1.27	1.12	1.38		1.89
P	0.35	0.06		0.22	0.28	0.20		0.10

TABLE 3—(*continued*)

Observer B								
Exp. 1								
M.D.	−2.65	8.8	−0.9	2.6	−0.2	7.5	4.7	11.8
S.E.	3.12	2.40	1.18	1.15	4.49	2.66	2.76	1.52
t	−1.92	3.67	−0.76	2.26	−0.11	2.82	1.70	7.76
P	0.08	0.00	0.46	0.06	0.91	0.02	0.12	0.00
Exp. 2								
M.D.	−0.65	1.5	−0.4	−0.2	0.10	1.9	−0.6	1.5
S.E.	1.44	1.05	0.40	0.48	2.67	1.29	0.43	1.79
t	−1.24	1.43	−1.00	−0.42	0.09	1.47	−1.40	0.84
P	0.23	0.18	0.34	0.68	0.93	0.18	0.20	0.42
Exp. 3								
M.D.	−0.75	2.6	0.0	1.1	1.65	1.2	0.0	4.5
S.E.	1.99	1.13	0.00	0.99	3.25	0.97	0.00	2.70
t	−0.99	2.30		1.11	1.10	1.24		1.67
P	0.33	0.04		0.28	0.27	0.24		0.14

Key to code-lettering is given in Table 1.
I-A—Inter-action between anterior pituitary extract and adrenalectomy.
M.D.—Differences of the average score of experimental group b, c, and d, each taken against the control group a, except in the case of the Inter-action where

$$\text{M.D.} = \frac{(a+d)-(b+c)}{2}$$

S.E.—Standard error.

duct stimulation) was noted in the glands of injected rats but not in those of the controls (Figs. 1 and 2). Stimulation of duct growth, to an extent which resulted in an increase in mammary gland area in the males, was a prominent feature of Experiment 2. Stimulation of alveolar growth was observed only in the oldest rats (males) and by one observer only ($P=0.04$).

The appearance of the glands of many of the males in Experiment 3 was reminiscent of that of the glands described by Astwood, Geschickter and Rausch (1937) in normal adult male rats or in gonadectomized immature rats of both sexes treated with androgens in that the duct systems remained relatively restricted in extent but were covered with dense clusters of alveoli (Figs. 3 and 4). It seems possible that in males, at least, the pituitary extract evoked the secretion of adrenal cortex steroids with the androgenic activity.

MAMMOGENIC EFFECTS OF ANTERIOR-PITUITARY EXTRACT IN GONADECTOMIZED-ADRENALECTOMIZED RATS

The results in Tables 2 and 3 indicate that the anterior-pituitary extract was capable of exerting mammogenic effects in the absence of both adrenals and gonads. It is thus quite certain that the integrity of the adrenal cortex is not essential for the exhibition of, at any rate, partial mammogenic effects of unfractionated anterior-pituitary extracts of this type. With regard to the question whether the response to the extract was diminished in the absence of the adrenals, the effect

of adrenalectomy was too inconsistent in this respect to make possible a clear study of its interaction with anterior-pituitary extract. None of the interactions was significant but in Experiments 2 and 3 there were a number of large negative values of "t" indicating that the effects of anterior-pituitary extract were often upset by adrenalectomy.

COMPARISON OF SCORES OF OBSERVERS A AND B

It is unnecessary to give details of the analyses of the scores of the two observers. It is sufficient to note that the differences between Observers A and B in their methods of ranking were significantly smaller than the natural differences between animals; in many cases they were negligible.

CONTINUED DEVELOPMENT OF THE MAMMARY GLAND IN THE ABSENCE OF THE GONADS

The scores for alveolar development in the mammary glands of the gonadectomized control rats (Table 3) show that alveoli which were absent from the glands of controls killed at 28 days of age were present in the glands of control groups of both sexes killed at greater ages. Thus, despite the absence of gonads, a new type of structure appears in the mammary gland as the animal matures. The development of alveoli under these circumstances may be due to the action of adrenal cortex steroids acting directly on the mammary gland or, possibly, via the anterior pituitary. In this connection it may be recalled that progesterone, which is well known to possess the property of promoting mammary alveolar development has been isolated from adrenal cortex extracts by Beall & Reichstein (1938) and this might well be the substance responsible for this phenomenon. Further studies of mammary changes in untreated rats in the absence of the gonads are in progress in this laboratory.

DISCUSSION

Our results on the effects of adrenalectomy on the mammary gland are not in agreement with those of Butcher (1939) and Reeder & Leonard (1944). It must be noted that our experiments were carried out under different conditions from those of the above-mentioned workers since Butcher used rats which were deliberately underfed, while Reeder & Leonard studied only one gland from each rat. Where any significant changes following adrenalectomy were observed in our experiments they were, in contradistinction to the results of the above-mentioned authors, always in the direction of regression as might be expected from the considerations advanced in the introductory paragraphs of this paper. It must be conceded, however, that such changes were never dramatic even in the oldest rats whose glands were best developed when the adrenals were removed. This work therefore provides little evidence that the adrenal cortex plays an outstanding and essential role in the normal development of the mammary gland.

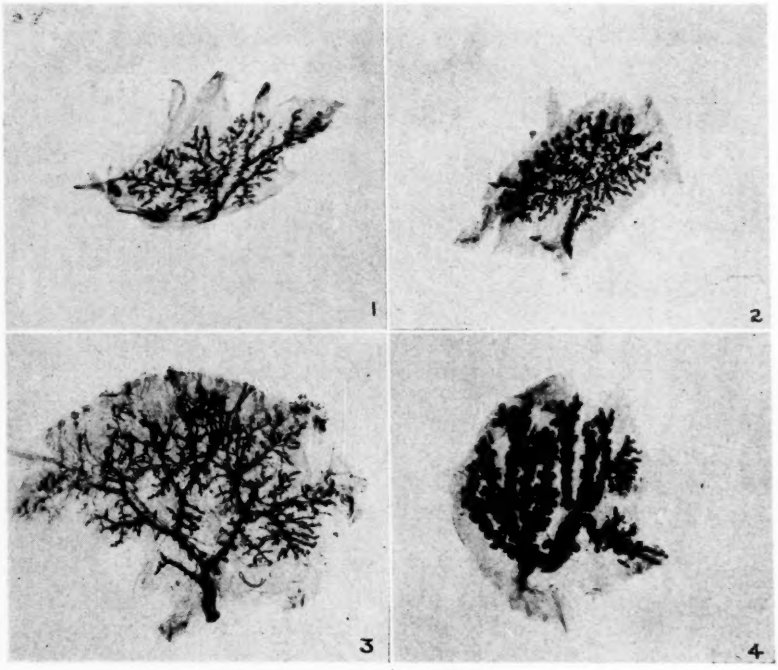

FIG. 1. Whole mount of 2nd thoracic mammary gland of untreated male rat gonadectomized at 21 days of age and killed at 28 days. ×5.

FIG. 2. Whole mount of 2nd thoracic mammary gland of male rat gonadectomized at 21 days of age and receiving six daily injections of anterior-pituitary extract immediately before autopsy on the 28th day. Note the club-shaped end-buds. ×5.

FIG. 3. Whole mount of 2nd thoracic mammary gland of untreated male rat gonadectomized at 27 days of age and killed on the 84th day. ×5.

FIG. 4. Whole mount of 2nd thoracic mammary gland of male rat gonadectomized at 27 days of age and receiving ten daily injections of anterior-pituitary extract immediately before autopsy on the 92nd day. The restricted duct system is covered with dense clusters of alveoli. ×5.

Our demonstration of mammary growth in response to anterior-pituitary extract in the absence of the gonads is in accord with the previous findings of Corner (1930), Lyons & Catchpole (1933–34), Asdell, Brooks, Salisbury and Seidenstein (1936) and Gomez & Turner (1937–38) in the rabbit and of Gomez & Turner (1937–38) and Greep & Stavely (1941) in the rat. We have evidence that the type of response evoked depends on the degree of glandular development existing at the time of treatment. This is to be expected since, although the extract evidently contained a factor or factors capable of causing both duct and alveolar development, the latter would probably not occur until the duct system had reached a suitable state of development, while duct growth factors could hardly be expected to cause further growth of a fully developed duct system.

Qualitatively similar mammogenic effects were often exhibited in the absence of the adrenals in addition to the gonads and while the response may have suffered some diminution in adrenalectomized rats the degree of such diminution was relatively slight. It is thus evident that adrenotropin could hardly have been an important participant in the mammogenic effect. The results in general add to existing evidence that anterior-pituitary extracts contain factors which can cause development of both mammary ducts and alveoli by direct action on the mammary gland, and it appears that they can do this without the aid of steroid sex hormones arising either from the gonads or the adrenal cortex.

In conclusion, a few remarks on the technique of studying mammary gland development in laboratory rodents may not be out of place. Many workers in carrying out such studies are content to examine only one gland from each animal. We are of the opinion that this practice may often give rise to misleading conclusions and, despite the considerable additional work involved, we consider it desirable to examine and score, by some such method as that described in this paper, all the glands from all the animals.

SUMMARY

A semi-quantitative method of studying changes in the mammary gland structure in the rat is described. It has been used to study the effects of adrenalectomy and of treatment with anterior-pituitary extract, singly and in combination, on the mammary gland in gonadectomized rats of both sexes.

Regressive changes were frequently, but not invariably observed following adrenalectomy; increased arborescence of the duct system was never observed. Ox anterior-pituitary extract exhibited mammogenic activity both in the presence and absence of the adrenals, stimulation of duct growth being most prominent in the youngest rats while in older rats stimulation of alveolar development also occurred. There was some evidence that the mammogenic effects of the anterior-pituitary extract were sometimes upset by adrenalectomy but the results indicated that the mammogenic action of the extract was not primarily due to the action of adrenal cortex steroids secreted in response to stimulation by adrenotropin.

Alveoli eventually developed in the mammae of untreated rats gonadectomized when sexually immature; this phenomenon may be due to the action of progesterone secreted by the adrenal cortex.

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ACTION *IN VITRO* OF THYROTROPIC HORMONE AND IODIDE ON THYROID CELLS

L. C. JUNQUEIRA, M.D.[1]

From the Institute of General Anatomy and Embryology, Faculty of Medicine

BUENOS AIRES

STUDIES of the cellular changes of the thyroid gland in the intact animal are complicated by the intervention of many factors such as innervation and blood supply, which might influence cell activity. The thyroid is also dependent on the action of other organs, principally the pituitary gland, which regulates thyroid physiology. For these reasons it seemed advantageous to use an *in vitro* technique by which such uncontrolled complicating factors could be avoided, thus permitting a study of the cytology of the gland under well controlled conditions. It was also thought that with this method the influence on the thyroid cells of different drugs and hormones known to affect the activity of the gland *in vivo* could be studied with a view towards determining if the action of the agent is exerted directly on the thyroid cells or indirectly.

In this paper we will describe the results obtained by using thyrotropic hormone and iodide. The action of thyrotropin *in vitro* has been studied by Eitel, Krebs and Loeser (1933), who described only histological changes. The use of the freezing-drying method, as applied to the thyroid by De Robertis (1941), has enabled us to study the cytological changes particularly those involved in the secretion and reabsorption of the colloid. It was found that thyrotropin produces *in vitro* changes similar to those noted *in vivo* (De Robertis, 1942). It was found furthermore that iodide interferes *in vitro* with the action of thyrotropin.

METHODS

White rats of both sexes weighing approximately 60 gm. were used. The animals were killed by a blow on the head, the thyroid gland dissected out immediately and put in a Petri dish containing a 0.9% NaCl solution at 37°C. The tissue was then cut with very fine scissors so as to obtain fragments weighing approximately 1 mg. each. Thereafter they were put in Carrel flasks H.8 (six fragments in each flask) containing 1 ml. of nutritive medium composed of $\frac{2}{3}$ of homologous serum and $\frac{1}{3}$ of 0.9% NaCl and kept in an

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[1] Fellow of the "Fundos Universitarios de Pesquiza" of Sao Paulo, Brasil working at the Institut of General Anatomy and Embryology of the University of Buenos Aires, Argentina. Actual address: Laboratorio de Histologia e Embriologia, Facultade de Medicine, Universidade de Sao Paulo, Brasil.

oxygen atmosphere as described by Parker (1938). In preliminary control experiments it was found that the thyroid tissue could be maintained in good condition for 96 hours in such a medium, as judged by microscopical examination after fixation in Helly's fluid and staining with H and E. Once this was established the following experiments *in vitro* were performed: (a) Incubation of thyroid tissue in the nutritive medium for periods of time varying from 30 minutes to 48 hours. This group served as controls. (b) Incubation of thyroid tissue for a period of 3 hours in a nutritive medium to which sodium iodide had been added in various concentrations. (c) Incubation of thyroid tissue for periods ranging from 30 minutes to 24 hours in a nutritive medium in which thyrotropin in different doses had been added. (d) Incubation of thyroid tissue for 3 hours in the above medium plus iodide, with the subsequent addition of 1 Junkmann-Schoeller unit of thyrotropin and further incubation for 90 minutes.

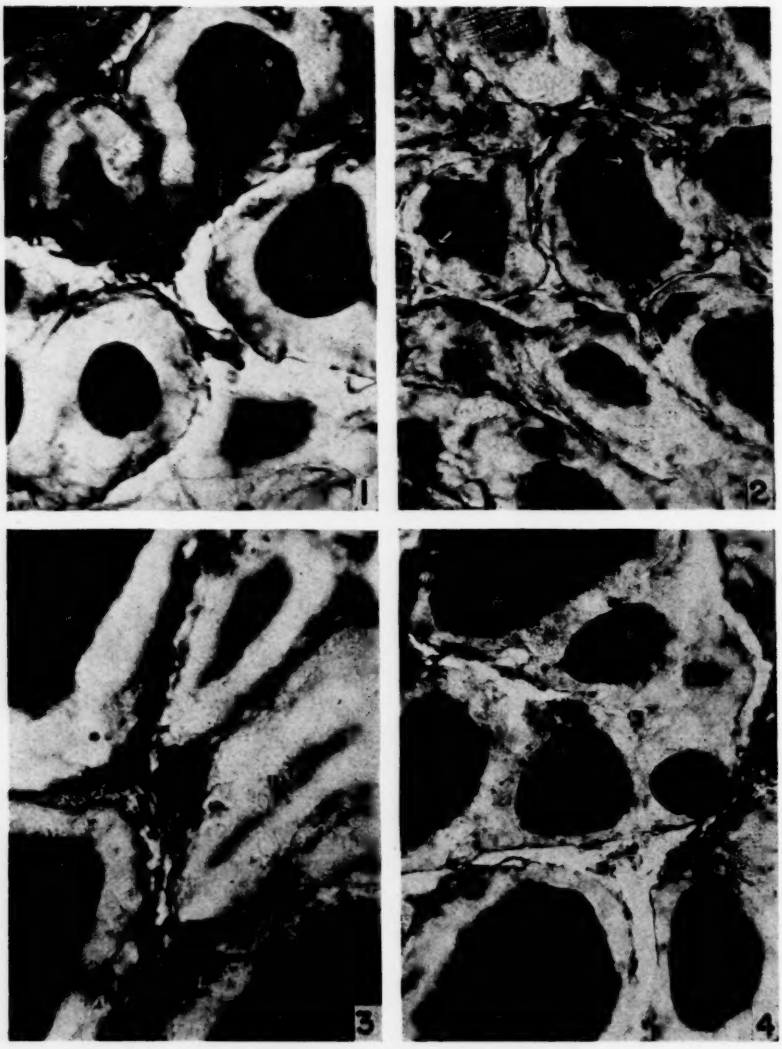
Sodium iodide was used in concentrations of 0.15 M to 0.00015 M. The dose of thyrotropin per flask varied from 1 to 10 J-S units. Immediately after incubation the tissue was frozen in liquid air and dried in vacuo at −30°C. (Gersh, 1932). Afterwards it was denatured in alcohol and stained to demonstrate the intracellular colloid according to the method of De Robertis (1941).

RESULTS

Action in vitro of thyrotropin on thyroid cytology. The addition of thyrotropin to thyroid fragments produced very marked cellular changes starting as early as 30 minutes. Even after such a short period of time the number of intracellular colloid droplets is greatly increased. These colloid droplets seemed at first to be secreted into the follicular cavity, since pictures of excretion similar to those described by De Robertis (1942) were found. As shown in Fig. 2, after 90 minutes of the action thyrotropin the apical zone of the cytoplasm of the thyroid cells became filled with large colloid droplets. Some of these droplets were being extruded into the follicular cavity and bulged toward the lumen surrounded by a thin rim of cytoplasm.

After longer periods of time reabsorption of the colloid became prominent and the thyroid cells came to be filled with the colloid released from the lumen. These two phases of secretion and reabsorption, however, were not so sharply defined as were the corresponding processes *in vivo* (De Robertis, 1942), and in many follicles both processes seemed sometimes to coincide. The colloid released from the follicles apparently remained in the interfollicular spaces and was taken up by macrophages. In Fig. 3 it is shown that after 24 hours of incubation with thyrotropin some follicles have released a great deal of their colloid. At this time thyroid cells were higher and had few colloid droplets, but macrophages of the connective tissue were filled with colloid droplets. It is interesting to recall that such macrophages are never seen in the gland *in vivo* under similar conditions.

Action of iodide on thyroid cytology. The addition of sodium iodide to the nutritive medium followed by incubation for 3 hours produced



All figures are from drying freezing fixed tissues stained with aniline blue-orange G. Red. filter.

FIG. 1. Rat's thyroid fragment of group *a* (normal control). Small quantity of intracellular colloid droplets. 900×.

FIG. 2. Rat's thyroid fragment of group *b* (incubated during 90 minutes with one Junkmann-Schoeller unit of thyrotropic hormone). Great increase of the number of intracellular colloid droplets, some of which are excreted into the follicle (arrows). 900×.

FIG. 3. Rat's thyroid fragment of group *b* (incubated during 24 hours with one Junkmann-Schoeller unit of thyrotropic hormone). Increase of cellular height. Great reab-

no definite changes in the thyroid cells, except that with strong concentrations a diminution in the number of colloid droplets took place.

Action of iodide and thyrotropin on thyroid cytology. When the action of thryotropin on thyroid cells in a medium containing sodium iodide was studied, striking results were obtained. The presence of iodide, especially in strong concentrations, (M. 0.15 and 0.015) produced a definite inhibition of the action of thyrotropin. This effect is shown in Figs. 2 and 4. In each case one Junkmann-Schoeller unit of thyrotropin had been added to the medium, but in the second case sodium iodide was also present. In this second case the cells appeared even lower than in the normal gland (Fig. 1) and only a few colloid droplets could be found in the cytoplasm. The processes of secretion and of release of the colloid, both of which were greatly increased by the action of thyrotropin, were almost entirely suppressed with the iodide.

DISCUSSION

Results reported here clearly demonstrate that the thyrotropic hormone of the pituitary gland acts directly on the thyroid cells and prove that neither innervation nor vascularization are necessary for this action. This conclusion confirms the histological reports by Eitel, Krebs and Loeser (1933) *in vitro* and also agrees with the results of Marine and Rosen (1934) who showed the stimulating action of thyrotropin on thyroid transplants. Our results do not support the hypothesis proposed by Dobrzaniecki and Aron (1930) that the cervical sympathetic chain is indispensable for the action of thyrotropin on the thyroid. This point of view has also been rejected by Krayer, 1933, Uotila, 1939, and by Loewe, Ivy and Brock, 1945.

Therefore the mechanism of action of thyrotropin seems to be mainly humoral although the possible association of a nervous factor cannot be completely ruled out, by these experiments. Such intervention might be suspected in view of the work of Pieper, 1934, who obtained less activation in denervated thyroids than in controls. However, these results need to be confirmed with better histological methods for estimating thyroid activity.

The cytological changes obtained with thyrotropin *in vitro* fully confirm the observations *in vivo* of De Robertis (1942) in the rat and in the guinea pig. It seems to us very interesting that the circulation is not indispensable either for the secretion or the release of the colloid, since both processes occur directly *in vitro*. However, in this case the

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sorption of intrafollicular colloid droplets with collapse of follicular walls. In the intrafollicular space several colloid droplets clumped together inside a macrophage are seen. 1200×.

FIG. 4. Rat's thyroid fragment of group *d* (incubated during 3 hours in nutritive media with 0.15 M sodium iodide plus 90 minutes with one Junkmann-Schoeller unit of thyrotropic hormone). Reduced number of intracellular colloid droplets. Compare with Fig. 2. 900×.

colloid released by the follicles is not transferred into the circulation, as in the intact animal, but remains in the interfollicular spaces where it is taken up by macrophages.

The *in vitro* action of thyrotropin is most intense and seems to start in the most peripheral follicles which are bathed directly by the nutritive fluid. The *in vivo* action is stronger in the internal follicles.

The fact that after 24 hours of the action of thyrotropin the number of colloid droplets in the cells is much lower than in the first few hours seems to agree with the report of Rawson, Sterne and Aub (1941) that explants of thyroid tissue, when bathed in a medium containing pituitary extract, inactivate the thyrotropic hormone.

Results reported here show very definitely that iodide in strong concentrations interferes *in vitro* with the action of thyrotropin. Iodide apparently inhibits the action of thyrotropin not only in the release of the colloid but also in the synthesis and secretion of the hormone. The actual mechanism of the inhibiting action of iodide is not known, but there are reasons to believe that it may act by blocking the enzymatic system of the thyroid gland on which thyrotropin acts. It may also act directly on the thyrotropin. De Robertis (1941) and Dziemian (1943) have demonstrated that thyrotropin activates the proteolytic-enzyme of the thyroid gland while iodide inhibits it.

These results together with those found in the therapeutic action of iodide in toxic goiter (De Robertis and Nowinski 1946 *a*) and the direct inhibition *in vitro* of the proteolytic enzyme by iodine (De Robertis and Nowinski 1946 *b*) suggest that iodide may act on this proteolytic system, thus interfering with the action of thyrotropin on the same system.

However, it has been suggested (Loeser and Thompson, 1934) that iodine may suppress the secretion of thyrotropin by the pituitary gland and that part of the action of iodine may be to prevent the action of thyrotropin (Friedgood 1936). Recent results reported by Rawson and col. (1945) strongly support the idea that iodide may act also by inhibiting thyrotropin.

SUMMARY

Fragments of the thyroid gland of young white rats have been maintained *in vitro* for as long as 96 hours and subsequently studied cytologically with the freezing-drying technique of Altmann-Gersh. The direct action of thyrotropic hormone and iodide was tested on these explanted preparations.

It was found that thyrotropin strongly increases the amount of intracellular colloid within 30 minutes of the time of application. It activates secretion of colloid droplets toward the lumen and release of intrafollicular colloid. The released colloid apparently remains in the interfollicular spaces and is taken up by macrophages. Sodium iodide in a concentration of 0.15 M to 0.015 M inhibits the action of thyro-

tropin on the secretion and release of the colloid. These results are discussed in view of recent findings bearing on the physiology and enzyme chemistry of the thyroid gland.

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NOTES AND COMMENTS

FLUCTUATIONS IN THE ESTROGEN LEVEL THROUGHOUT THE MENSTRUAL CYCLE OF ONE WOMAN

IN THEIR article on the use of the weanling guinea pig as a test object for estrogen assay, Hartman, Littrell and Tom (1946) reported that exploratory work indicated castrated guinea pigs are also exceedingly sensitive to minute doses of synthetic estrogens or estrogen in female blood. Further investigations by these authors show that if the spayed female guinea pig is primed with estradiol, opening of the vaginal closure plate can be induced with injections of 0.04 cc. of normal untreated female blood.

The work of Markee and Berg (1944) demonstrated the estrogen level in the blood of women throughout the entire cycle. The resultant curve is the condensed data obtained from assaying the blood of 75 women. (Fig. 2, heavy line.) Hamilton (1946) found that through the menstrual cycle of the monkey there was a variation in the height of the cervical glands which apparently fluctuated with the changes in the blood estrogen level. (Fig. 2, dotted line.) These data agree with the results of Markee and Berg. However, the methods employed by Markee and Berg could not conveniently be carried out on one individual, since their method necessitated the withdrawal of 50 cc. of blood by venipuncture. This procedure would seem a little drastic for daily use.

This paper represents an attempt to determine, theoretically at least, the daily estrogen level in the blood of one individual by the use of the method described by Hartman and Littrell (1945). One reliable graduate student 23 years of age, working in this laboratory, agreed to regular daily donations of blood throughout her cycle, and the experiment was begun just before menstruation. It is regrettable that the cycle in question happened to be one of the subject's shortest, namely 22 days.

Female guinea pigs that had been castrated 30–60 days previously were used as the test object. The animals were standardized by a priming dose of 0.2γ of estradiol 5 days before the blood was administered. This dosage was later found to make the animal too sensitive and the priming dose was reduced to 0.1γ.

A total of 54 guinea pigs were used in the course of this experiment, 3 animals being injected each day of the cycle with 0.04 cc. of freshly obtained blood. Lack of a sufficient number of castrated animals necessitated using over again those injected in the early part of the cycle. Hourly observations were made over a period of 25 days and nights, and the time of vaginal opening recorded.

The bar graph (fig. 1) demonstrates the actual time of vaginal opening. Each bar represents the results of one day, and is obtained by taking the average time of vaginal opening of the 3 animals injected that particular day.

A curve was then constructed (dotted line) which conformed to the vari-

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ation in heights on the bar graph (fig. 1). Since it has been demonstrated that the time of vaginal opening is inversely proportional to dosage of estrogen given, this can be taken as the curve of vaginal opening. Therefore the minimum on the constructed curve would indicate a higher blood estrogen

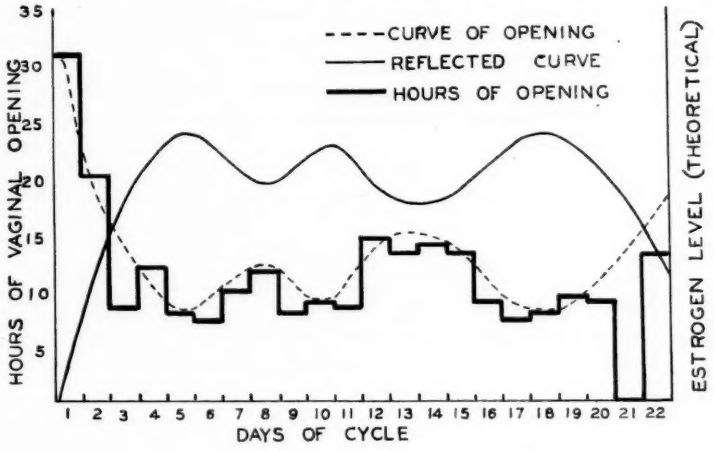

FIG. 1

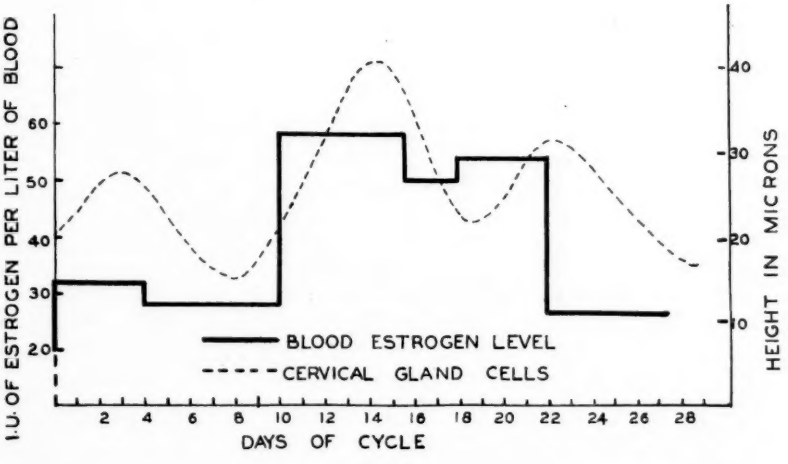

FIG. 2

level, and in the same manner the maximum would indicate a lower estrogen concentration. A reflected curve (solid line) was then drawn which can be said to demonstrate the theoretical blood estrogen level throughout the entire menstrual cycle of one individual. When this curve is compared to the graph of Markee and Berg (fig. 2) and the curve of Hamilton (fig. 2), it is seen that

there is an inclination of the reflected curve to follow the same fluctuations in estrogen level.

The authors readily admit to the inadequacies of this experiment. The number of animals used daily should be more than was used here, and there should be enough castrates in readiness so that none need be used more than once in a given cycle. (The castrated guinea pigs if allowed to regress after an experiment can be used many times. Some of the castrates in our colony have been in use for 10 months.)

A standard curve should be constructed by plotting the time of vaginal opening against a known amount of estrogen. With such data the actual level of blood estrogen each day of the menstrual cycle could be easily evaluated. This experiment is offered only as an interesting indication of the possibilities in the study of the cyclic variations of estrogen in individual cases.

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JAE L. LITTRELL
JOHN Y. S. TOM

From the Department of Zoology and Physiology,
University of Illinois,
Urbana, Illinois

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LACTOGENIC HORMONE AND FAT METABOLISM

PERIPHERAL fat stores of lactating rats are always very reduced. The body weight shows a tendency to fall. A causal relationship was suspected between an abundant circulation of lactogenic hormone and the reduction of the fat stores, at a time when fat globules were undoubtedly appearing in the mammary epithelial cells.

On looking through our clinical case histories of adiposities in the female, we found a number of instances where the weight curve had risen sharply for the first time after the delivery of a child that had to be artificially fed owing to sheer poverty of the maternal milk supply. These cases of adiposity were typically refractory to thyroid medication even when pushed to the upper limit of tolerance.

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It was considered that the development of this form of adiposity might be causally related to insufficient secretion of lactogenic hormone by the anterior pituitary.

Lactogenic hormone fractions were prepared by repeated reprecipitation at the isoelectric point, using as starting material crude pituitary anterior lobe extracts, prepared by acid acetone extraction in accordance with the procedure of Lyons (1). The preparations used were found to contain a minimum of 7 I.U. and a maximum of about 22 I.U. lactogenic hormone. Doses up to 22 mgs. did not show any gonadotrophic or thyrotrophic activity. Corticotrophic hormone was present only in traces, 30 mgs. containing one sudanophobic unit.

Eight thyroid-resistant cases of adiposity dating from lactational failure were treated with daily injections of 100–150 units of this material. The first signs of response appeared after the fourth injection, when the women felt much better—largely because their skin no longer felt uncomfortably tight, and the weight curve stopped rising. The next sign was a real fall in the weight curves, to a maximum of 7 lbs. within a fortnight. If at this stage the cases received thyroid extract in addition, a considerable weight loss would continue to occur no matter how thyroid-resistant the women had been in the past. Steady losses at the rate of 14 lbs. a month were by no means unusual and might be continued for at least six weeks after the lactogenic hormone had been withdrawn and thyroid only given.

A wide variety of cases of adiposity whose history had not been necessarily at all suggestive of lactational insufficiency were then treated. It was of particular interest to watch the effect of the lactogenic hormone upon types whose rate of weight increase had been rather alarming, as in acute Cushing's syndrome or in certain cerebral disorders attended by abnormally big appetite. The presence of red striae showed where fat deposition was still proceeding more quickly than the skin could grow. Consistently these red striae lost their red colour within the first fourteen daily injections. Consistently also these patients voluntarily reported loss of the very tight feeling they had had, particularly over the abdomen. In one particular case of adiposis dolorosa (Dercum's syndrome) the tightness had amounted to considerable pain, but the phenomena had disappeared completely by the end of the fourth week of daily injections of 400 units lactogenic hormone. With the addition of thyroid gr.V daily, this case lost 70 lbs. in four months.

A biological analysis of the mechanism was then begun, using the Wistar rat as a test object. The total fat content was determined—

(a) in the stripped skin, and

(b) in the carcass after removal of the intestines.

The method of determination of total fat content was to digest the skin or the carcass with five times its weight of 30% KOH on a boiling water bath with frequent shaking for 3 hours. The resultant solution was then cooled, transferred to a graduated flask, and filled up to the mark with water. 25 or 50 ml. was transferred to a separating funnel, acidified to litmus with 40% sulphuric acid, and extracted four times with petroleum ether. The combined extracts, after washing with distilled water, were dried overnight with anhydrous $Na_2 SO_4$, then evaporated in a weight beaker by blowing warm air over the surface, and finally drying by evaporation in a vacuum dessicator. The fatty acid content was determined by weighing the residue.

Table 1 shows the fat content of skins of infantile female and male rats,

and adult female and male rats, after six days' treatment with lactogenic hormone. The skins of each control and treated group of animals were always dated and investigated together. The values quoted are therefore the average values of ten animals. The skin fat content of all the treated animals

TABLE 1

Number of rats in group	Sex	Average body weight in gr.	Treatment	Fat content of the skin in %	Changes seen in treated animals compared with control group
10	♀	35	Controls	8.4	
10	♀	36	Injection of 0.4 mg. Prolactin (circa 3 I.U.) daily for 6 days.	3.4	−59%
10	♂	42	Controls	7.2	
10	♂	40	Injection of 0.4 mg. Prolactin (circa 3 I.U.) daily for 6 days.	4.5	−37%
10	♀	145	Controls	22.8	
10	♀	148	Injection of 2 mg. Prolactin (circa 14 I.U.) daily for 6 days.	14.05	−38%
10	♂	137	Controls	18.5	
10	♂	134	Injection of 2 mg. Prolactin (circa 14 I.U.) daily for 6 days.	10.6	−42%

TABLE 2

Number of rats in group	Sex	Average body weight in gr.	Treatment	Fat content of skin %	Fat content of carcass %
5	♀	85	Control fed normally	16.1	4.8
5	♀	86	Fed normally. Injected daily with circa 22 I.U. Prolactin for 5 days.	12.9	4.3
5	♀	55	Starved for 5 days.	3.5	2.9
5	♀	48	Starved for 5 days. Injected with 22 I.U. Prolactin daily.	1.8	2.3

of all four groups was considerably decreased, the decrease varying from 37% to 59%.

Attention might here be drawn to the fact that the fat content of the skin of rats was invariably found to be higher in the female than in the male.

Table 2 shows the result of an experiment investigating the fat content of normally fed rats, and rats starving for five days, without and with prolactin treatment. The normally-fed rats lost about 20% after prolactin treatment and the starving rats also lost fat in their skin. The group which was starved

and was treated with prolactin simultaneously showed a skin fat content 60% lower than the group which was starved only.

The fat content of the carcass is much less reduced after treatment with lactogenic hormone than that of the skin.

Table 3 shows the effect of combined treatment with lactogenic and thyroid hormones. The fat content of the skin after thyroid treatment is

TABLE 3

Number of rats in group	Sex	Average body weight in gr.	Changes in body weight during experimental period	Treatment	Fat content %	
					Skin	Carcass
6	♀	182	+ 2 g.	Controls	21.8	6.0
6	♀	180	− 6 g.	2 gr. Thyroid daily for 7 days.	20.9	5.7
6	♀	180	− 2 g.	Prolactin circa 30 I.U. daily for 7 days.	15.6	5.3
6	♀	168	−14 g.	2 gr. Thyroid+circa 30 I.U. Prolactin daily for 7 days.	10.7	4.1

TABLE 4

Number of rats in group	Average body weight in grs.	Treatment		Injected prolactin dose in mg.	Fat content of skin %
8	48	All animals starved for 48 hours.	Untreated controls.	0	5.39
8	49		Animals receive one injection 24 hours before dissection.	1	4.73
8	42			4	2.82
8	44			8	3.18
8	40			16	1.81

only inconsiderably decreased (in other experiments we found no decrease at all, sometimes even a slight increase). After prolactin alone, the skin fat is reduced by about 37%, but after prolactin and thyroid treatment the fat content fell by over 50%. Changes in the same direction but on a lower level were found on investigating the carcass.

Table 4 shows the results achieved after different doses of prolactin were injected 24 hours before dissection of animals starved for 48 hours. It is interesting that, even after such a short interval, very characteristic changes in the fat content were found; changes which are to a certain extent proportional to the dose given.

The fat content of the liver was also investigated in many experiments, but no consistent results were obtained. The values found are therefore omitted from the tables.

DISCUSSION

Assuming that the action described is really due to the lactogenic hormone, and not to an active substance consistently accompanying that hormone through all the manifold purification procedures, it might be considered as probable that lactogenic hormone mobilises fat from the peripheral fat deposits.

The fat, lying sometimes in quite solid conglomerations in the subcutaneous cell tissue with poor blood circulation, is apparently mobilised by the lactogenic hormone, migrating to places where it is metabolised, as for instance in the liver, or excreted, as in the mammary gland. Once mobilised, thyroid hormone can increase its metabolism, hence the accumulative activity of thyroid hormone and lactogenic hormone. From the pathophysiologic point of view, thyroid resistance of certain adiposities becomes understandable, as does also the development of the adiposity after confinement.

SUMMARY

The fat content of peripheral fat deposits is reduced after treatment with lactogenic hormone. This was shown on rats where the total fatty-acid content of skins was investigated, and in clinical cases of adiposity where red striae disappeared shortly after the commencement of the treatment.

Thyroid treatment alone has no effect, but increases the action of the lactogenic hormone.

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MAX REISS

From the Biochemical and Endocrinological Research Department of Bristol Mental Hospitals